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# THE INFLUENCE OF THE THYROID IN MALIGNANT DISEASE.

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## REGIONAL AND LOCAL DIFFERENCES IN CANCER DEATH RATES.

REFERENCE to the table of incidence of malignant disease in various countries (Table I) tends to confirm the work of earlier workers in the field of the demography of cancer. These figures, recently published by World Health Organisation (1952*a*), are fully standardised, i.e., corrected for age and sex differences in the population of each country, and so are as comparable one with another as it is possible to make them. All investigations on these lines during the past half century have pointed to the same conclusion: that the incidence of malignant disease is largely conditioned by local factors, and Stocks (1947), after a lifetime of work on this problem, can do no better than quote Hoffman's (1915) statement made 40 years ago, that "the local variations in cancer frequency throughout the world are primarily conditioned by local causes and not by faulty methods of diagnosis or defective methods of death registration." More recent work by Stocks (1950) and by Legon (1951, 1952) has given further detail to the position in this country. The present situation has been briefly restated by Stocks (1953) at Cardiff.

There have been many endeavours to establish a racial factor to account for the known variations in cancer incidence. Although they have been invariably inconclusive, as might be expected when consideration is given to the pronounced differences within individual countries, these surveys have shown that where racial types have migrated to other countries, e.g., negroes to America, they appear to take over the incidence of malignant disease of the natural inhabitants among whom they have come to live. At the International Cancer Conference in London in 1928, Sourasky (1928) was able to show that although the Jews have certain cancer characteristics regarding individual organs, their overall mortality from cancer in different parts of the world varies and tends to approach that of the non-Jews among whom they come to live. Nordling (1953) comments on the great difference in the cancer frequency between Whites in the north-eastern United States and those living in the southern part of the country, as well as between the negroes in those two parts; and concludes that environment rather than race appears to be responsible for those differences. This finding has a further bearing on the data discussed below in the section dealing with the United States of America.

Such positive evidence as there is, seems therefore to lend support to the hypothesis of a geographical rather than a racial factor to account for the observed variations in the incidence of malignant disease as set out in Table I.

TABLE I.—*Standardised figures for Cancer Incidence.*

(World Health Organisation.)

Percentage of all deaths statistically attributed to malignant tumours.

| | 1901. | 1920. | 1947. |
|---|---|---|---|
| *European countries.* | | | |
| Germany | 3·68 | 5·78 | 14·95 |
| England and Wales | 4·44 | 9·37 | 15·06 |
| Belgium | 3·44 | 5·38 | 10·12 |
| Denmark | — | 10·51 | 16·19 |
| Scotland | 4·57 | 8·46 | 13·70 |
| Spain | 1·53 | 2·54 | 5·91 |
| Finland | — | 4·49 | 10·09 |
| France | 3·51 | 4·71 | 11·90 |
| Ireland (Republic of) | 3·41 | 5·69 | 8·99 |
| Italy | 2·40 | 3·68 | 8·50 |
| Norway | 6·39 | 8·58 | 15·32 |
| Netherlands | 5·44 | 9·39 | 16·41 |
| Portugal | 1·16 | 1·15 | 3·59 |
| Sweden | 7·61 | 8·59 | 12·99 |
| Switzerland | 7·12 | 9·26 | 15·74 |
| *Extra-European countries.* | | | |
| Union of South Africa | 4·53 | 5·31 | 12·49 |
| Canada | — | 7·13 | 13·27 |
| Chile | 1·04 | 1·30 | 4·82 |
| United States | 4·05 | 6·42 | 13·13 |
| Uruguay | 3·96 | 5·24 | 14·79 |
| Japan | 2·38 | 2·85 | 4·66 |
| Australia | 5·18 | 8·01 | 12·88 |
| New Zealand | 6·75 | 8·50 | 14·56 |

## GEOGRAPHICAL SURVEY OF CANCER AND GOITRE INCIDENCE.

Endemic goitre, in varying degrees of severity, has a world wide and distinctive distribution, fundamentally based on iodine distribution or at times on iodine availability, and it is not difficult to show that iodine deficiency and cancer incidence share much common ground. It must be emphasised that endemic goitre is no more than a convenient indicator of iodine deficiency, which in itself is nowhere considered as a cause of malignant disease but only as an important contributory factor in the susceptibility to cancer rather than its cause.

### (1) *Low countries.*

Holland and Belgium are comparable countries of similar size, geographical aspect, wealth, standard of living and racial background. Belgium has however, a crowded population of far greater density than Holland and much heavy industry, features commonly associated with a high cancer incidence; whereas Holland has notably less urbanisation and a population that is considerably more agricultural. The cancer incidence of Belgium is only10·12 and goitre is not common, except in the south towards the Ardennes and near the Luxembourg border, and this comparative freedom from goitre is associated with a moderately high content

of iodine in the drinking water throughout most of the country (McClendon, 1939 ; Clinquart, 1926). By contrast, Holland has the highest cancer incidence of any country in the world, 16·41, and a notorious goitre problem that is centuries old. The accompanying map (Fig. 1) of 1953 indicates in black the areas where goitre is considered sufficiently common by the authorities to warrant the compulsory addition of iodised salt to the bread. Since this map was drawn up last

FIG. 1.—Goitre distribution in Holland (Amsterdam University).

year, three further districts have been added to the list in the Northern Province of Holland (Polak, 1954 ; personal communication). The lower incidence of goitre towards the Belgian frontier is apparent and almost certainly significant.

(2) *United Kingdom.*

Variations of goitre and cancer within the United Kingdom are striking. A recent survey (Stocks, 1950) of the incidence of carcinoma of the stomach in 83 county boroughs over a period of 18 years showed notable differences " which cannot be explained by chance variation or by differing accuracy of certification of cause of death". These variations were shown to correspond to variations in the hardness of the water supply : those with a moderate hardness tended to have

a lower incidence of cancer than towns with soft or very hard water. The Medical Research Council Goitre Survey of the United Kingdom (Murray, Ryle, Simpson and Wilson, 1948) showed that moderate hardness of drinking water is associated with less goitre than places with either soft or very hard water.

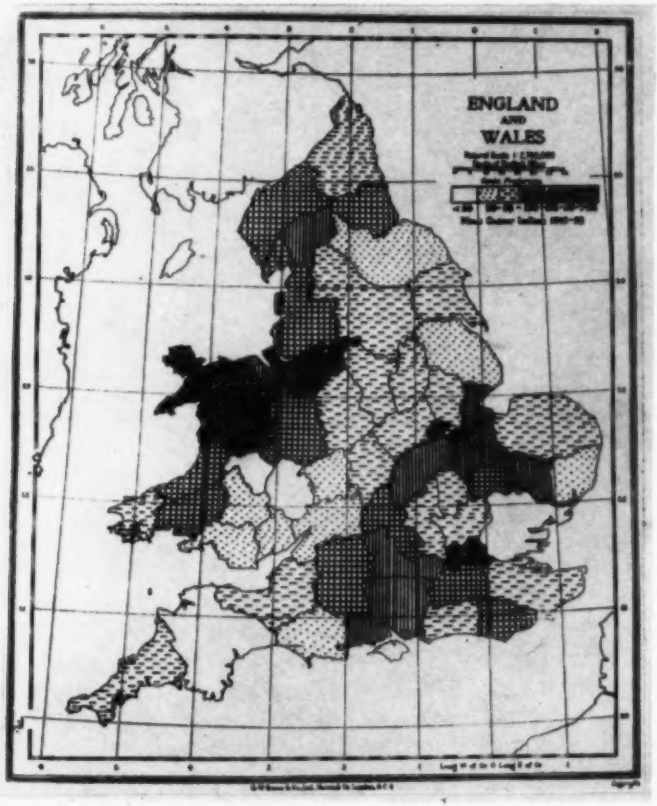


FIG. 2.—Mean cancer incidence for all sites, male and female (after Stocks, 1928).

The same survey showed less goitre in Scotland than in England and this is reflected in the lower cancer incidence rate for Scotland, 13·07, as compared with England and Wales 15·06.

In 1928 a map (Fig. 2) was prepared for the London International Cancer Conference by Stocks (1928) showing the incidence of cancer of all sites county by county. A few years later, McEwan (1938) produced a goitre map of England on a year's figures of deaths from toxic goitre (Fig. 3). Wayne (1954) has shown that toxic goitre and endemic goitre have similar distribution, and maps drawn from figures of toxic goitre have much advantage over those made from

surveys of recruits and schoolchildren, for these are necessarily incomplete and sporadic, and McEwan's map (Fig. 3) has a close resemblance to other maps of goitre (Campbell, 1925, 1927; McEwan, 1948). Certain anomalies have arisen, probably due to taking into account only one year's figures, for Oxfordshire is shown by Campbell's maps and the recent M.R.C. Survey of Goitre (Murray *et al.*, 1948) to have a high goitre incidence. This would be in keeping with the known clinical experience with regard to malignant disease in that county.

Comparing these two maps a striking correlation is at once apparent. Outstanding are the heavy incidences of goitre and cancer in mid-Wales, Westmoreland

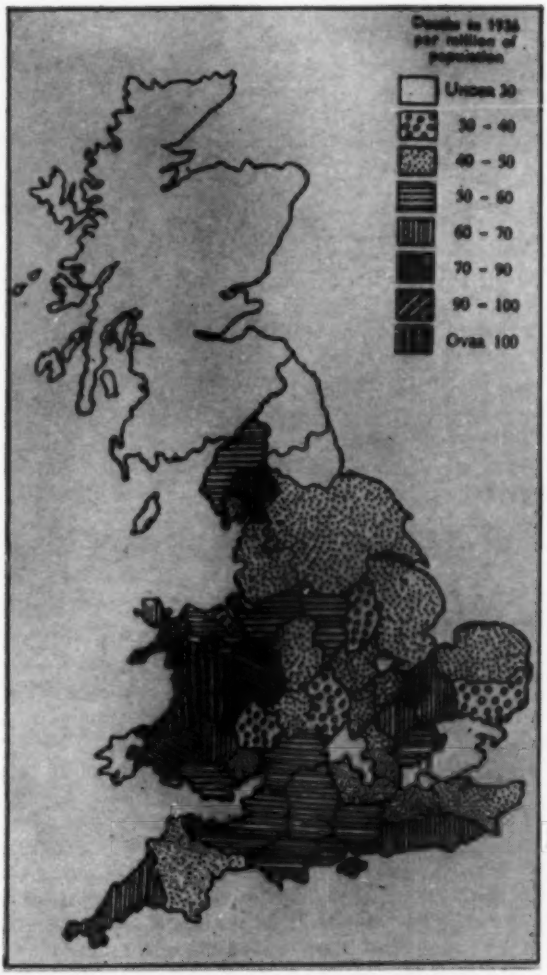


FIG. 3.—Goitre incidence derived from deaths from toxic goitre (after McEwan, 1938).

and the Fen district and neighbouring counties, and by contrast the light incidence in Yorkshire, Lincolnshire, Herefordshire and Pembrokeshire. Gradations of correspondence on both maps are evident in comparing Norfolk, Suffolk and Essex ; Kent and Sussex ; Cornwall and Devon ; Glamorgan and Carmarthen.

(3) *Sweden.*

A higher death rate from cancer was shown to exist in two Swedish counties, Kopparberg and Gefleborg, than was found in the total rural areas of Sweden (Stocks, 1925). Those two counties, as shown by examination of schoolchildren, recruits for the army and candidates for confirmation, were found to have a higher incidence of goitre as compared with the rest of the country.

(4) *Norway.*

Similarly, known goitrous counties in Norway were compared by Stocks (1924) with counties showing little goitre, and it was found that the goitrous counties showed a cancer rate for all organs of 113·7 as compared with 94·4 for the non-goitrous counties.

(5) *Iceland.*

In Iceland, the thyroid has been shown to contain one of the highest concentrations of iodine, and endemic goitre is non-existent (Rundle, 1951). Thyroids there weigh on an average 14 g. in men and 11·5 g. in women, as compared with the average of 25 g. in most countries (Kelly, 1946). Now Icelanders are mostly of Norwegian stock with some Danish admixture, and cancer incidence in Norway is 15·32 and in Denmark 16·41, but in Iceland it is only 5·98 (not standardised) (W.H.O., 1952*b*).

(6) *Spain and Portugal.*

Goitre is only mentioned as occurring in one province in Portugal, Alemtejo, on the southern end of the Spanish border (Kelly, 1946). Goitre is common in Spain where there has been a goitre commission for many years. The cancer incidence in Spain is 165 per cent of that found in Portugal.

(7) *Switzerland.*

Switzerland has always had a notorious goitre problem, but in spite of the absence of heavy industry and large towns, and in spite of its reputation for healthy living conditions, this country has always had a high incidence of malignant disease. Stocks (1924) found a close statistical relationship between the goitre rate of the various cantons as calculated from figures taken from the examination of recruits for the army, and carcinoma of the stomach and oesophagus of the general population. When considered canton by canton, the regression figure is $0{\cdot}6459 \pm 0{\cdot}0302$, or if the canton of Ticino is omitted the figure rises to $0{\cdot}7481 \pm 0{\cdot}0554$, which lifts the correlation well beyond the bounds of mere chance. Taking into consideration the lightly affected regions as well as the heavily affected ones it is possible to make out a correlation by inspection, though by no means so clearly as for England and Wales (Fig. 4, 5).

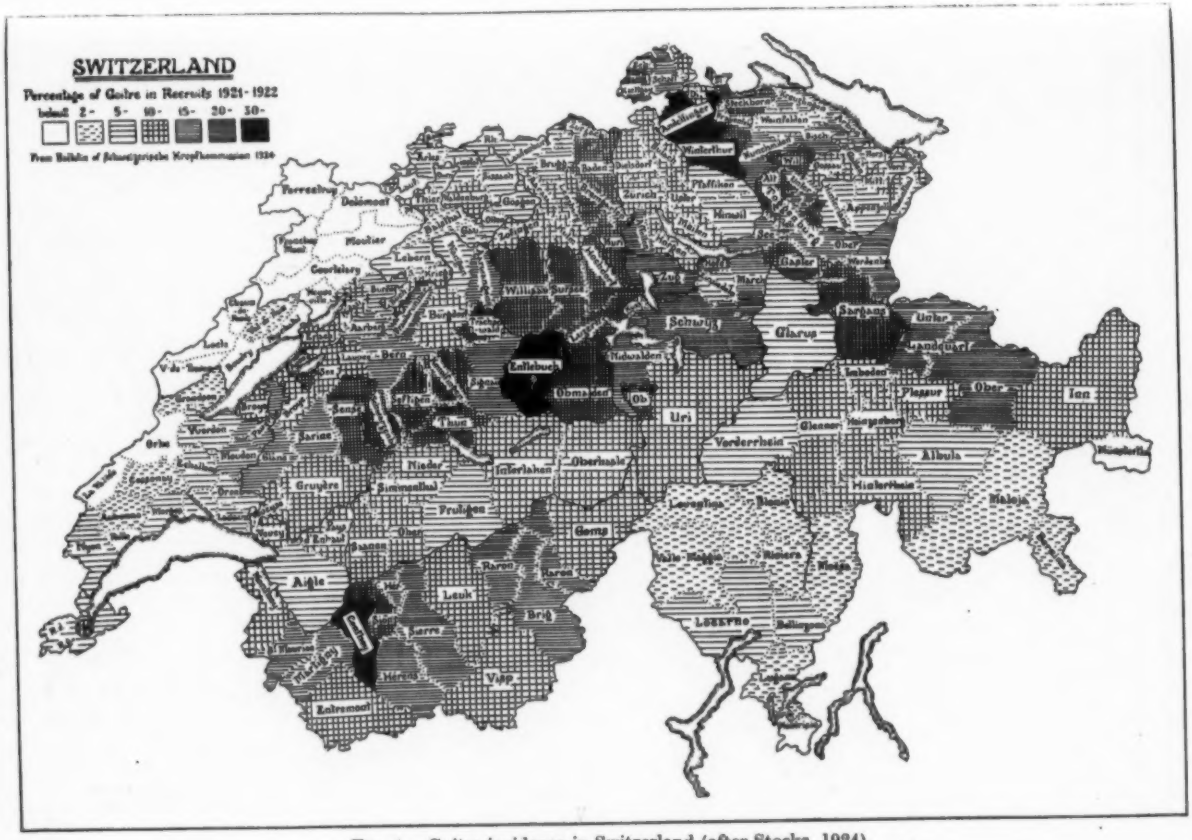


FIG. 4.—Goitre incidence in Switzerland (after Stocks, 1924).

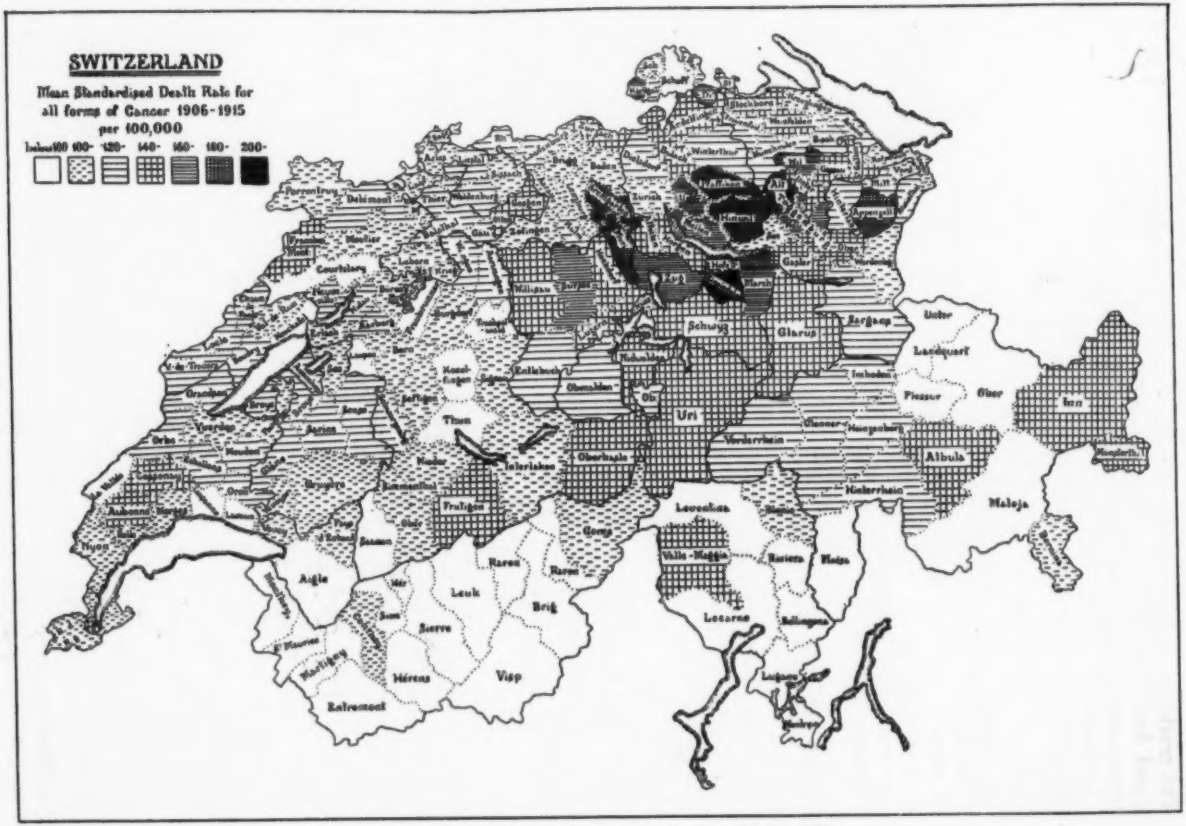


FIG. 5.—Cancer incidence in Switzerland (after Stocks, 1924).

(8) *Germany.*

The crude death rate for carcinoma of the lung among the miners of Schneeberg and Joachimsthal (where the radium for Madame Curie was first mined) is 25 times—not per cent—that of the general rate for carcinoma of the lung for the rest of Germany (Machle and Gregorius, 1948). These mines are fairly close together in one of the four main goitre centres of Germany on the Erzgebirge frontier of Saxony and Czechoslovakia. In these mines all the miners are presumably equally at risk from the inhaled radio-active material, but by no means all the men develop cancer of the lung and it is at least open to speculation that the apparent immunity of a considerable section of the mining community may be due to some factor associated with the presence or absence of goitre, or alternatively to a properly or feebly functioning thyroid. In this connection it is perhaps worth noting that a high incidence of lung cancer has not, so far as I am aware, been reported from other radio-active mines.

(9) *United States of America.*

The large goitre belt in the U.S.A. across most of the northern states and running south along much of the Pacific Coast (Fig. 6), first came to the public

FIG. 6.—Goiter incidence of U.S.A. (after McClendon, 1939).

notice during the Great War when, of nearly 12,000 men turned down for military service on account of goitre, some 3,600 were unable to button the military tunic around their necks on account of the size of their goitres (McClendon, 1939). If the forty large towns and cities of the U.S.A. are arranged in descending order

of their crude death rates for cancer as given by Hoffman (1915) it will be found that eight of the first ten lie in known goitrous areas, and that eight of the last ten (showing the lowest cancer rates) lie in the non-goitrous areas. Stocks (1925) has showed that there is a statistical correlation, with a regression figure of 0·644 ± 0·065, between the incidence of goitre, as recorded by the recruit figures, and carcinoma of the stomach and oesophagus, when considering separate states.

(10) *Australia and New Zealand.*

Australia is considerably more industrialised than New Zealand and has many large cities as compared with the latter country. Furthermore, rodent ulcer is infinitely more common in Australia, where goitre does not obtrude as a major health problem, though there are certain areas where there are definite, if small, districts where goitre is found (Kelly, 1946). The Australian cancer death rate is 12·88. The more rural and agricultural population of New Zealand has a death rate from cancer of 14·56. Here goitre is a major health problem and its incidence in Europeans, Maoris and animals alike has been closely studied for years. The Maoris are said to have a word, " tenga ", which has been in their language for centuries and means goitre (Kelly, 1946).

Fig. 7.—World goitre map (after Kelly, 1946).

(11) *Japan.*

In Japan much seaweed and fish is eaten, and goitre is rarely found except for a few cases in the central mountain areas (McClendon, 1939). The islands are densely populated, Tokio and Osaka having greater populations than any town in the United Kingdom except London, and there is much heavy industry and generally a low standard of living. These are conditions usually associated with

a high cancer incidence, but for long Japan has enjoyed one of the lowest cancer rates of the world, 4·66 in 1947.

This geographical survey lends support to the thesis not only that the general incidence of malignant disease is to a large extent conditioned by local factors, but that the one factor in evidence is apparently linked with the availability of iodine, using goitre as a clinical indicator.

## CLINICAL OBSERVATIONS.

A self evident association between cancer and goitre or impaired thyroid function from the clinical aspect is unlikely to have remained undetected till now. Although the substance of this enquiry took origin from certain clinical observations, data as clear as the geographical and experimental evidence cannot be expected for some years' time, when the results of the clinical trials and investigations that have been started become available. There are, however, a number of observations that, while not fully conclusive, may provide pointers sufficiently circumstantial to warrant further clinical investigation on the lines outlined here.

(1) The metabolic rate in man decreases progressively with increasing age, and the incidence of malignant disease increases progressively with age. The incidence of malignnat disease thus seems to be correlated with the decrease in metabolic rate. As metabolic rate is in general a rough indication of thyroid activity, there would be appear to be some relationship between malignant disease and thyroid dysfunction. Thus, the lowered metabolic rate often seen in diabetes mellitus (Joslin, 1946) may be considered with the increased liability of diabetics to develop carcinoma in any site or organ as compared with non-diabetics (Joslin, 1946). In the same way, the lowered metabolic rate commonly found in patients with all types of peptic ulcers (Kolmer, 1949) may be considered with the incidence of carcinoma of the stomach—still one of the commonest neoplasms of man.

(2) It has long been observed that puberty and pregnancy are periods when goitres may appear (Marine and Lenhart, 1909 ; Marine, 1935), and may disappear when the period of excessive demand ceases or when iodine is given. These are also the periods when melanomata tend to increase in size and may, not infrequently, become malignant. At the beginning of this year Mrs. R— was admitted to this hospital on account of malignant melanoma with spread to glands in the course of her third pregnancy. In her two previous pregnancies she also developed malignant melanoma on each occasion and was in this hospital in consequence. She has a moderate enlargement of the thyroid of the smooth " colloid " type seen commonly in pregnancy. The end of pregnancy is also the period when chorionic carcinoma and lactation carcinoma of the breast occur—perhaps the most rapidly growing class of cancer known.

(3) Administration of thyroid has been found to improve the action of oestrogens in the treatment of carcinoma of the prostate, permitting the effective use of smaller doses of oestrogen and delaying the onset of insensibility to oestrogen (Winsbury White, 1948). In support of this clinical experience a paper in endocrinological research is quoted (Chu and You, 1945), but the final conclusions of these workers was that the simultaneous administration of oestrogen and thyroid was the same as that obtained by thyroid feeding alone.

(4) Reduction of keloid formation can often be brought about by administration of thyroid. A series of such cases is given by Updegraff (1933), all in his

series showing a low metabolic rate. Although keloids are not neoplasms in the strict sense of the word, they tend to recur after adequate excision, and partly ulcerated keloids are prone to undergo carcinomatous change after a period of time.

(5) Weights of the thyroid of children coming to autopsy between the ages of 6 and 10 were observed in three European towns, and the cancer incidence of those noted (Bayard, 1919).

| | Bern. | Munich. | Kiel. |
|---|---|---|---|
| Average thyroid weights | 18·5 g. | 11·9 g. | 7·4 g. |
| Cancer mortality per 100,000 population | 132 | 98 | 57 |

(6) The iodine content of the thyroid has been shown to reach its maximum in the adult and remains fairly contant until the age of about 50, after which there is a gradual decline (Rundle, 1951). The age for the start of the decline in iodine content of the thyroid coincides in general with the age at which malignant disease in man becomes a prominent feature.

(7) In the second week of January of this year there were at least three patients in this hospital at the same time with gross goitre and cancer.

Mrs. M—, carcinoma of breast and a large colloid goitre visible seven or eight beds away down the ward.

Mr. E—, lymphosarcoma and a thyroid that weighed 80 g. at autopsy excluding involvement with growth.

Mr. R—, carcinoma of parotid. The nodular goitre caused some difficulty during resection of glands of neck.

Since January other patients have been seen in the wards of the hospital with coexistent carcinoma and goitre. It is relevant to note that the area from which this hospital draws its material, Gloucester, Somerset and Cornwall, is not one in which goitre is frequent.

(8) The association of goitre and malignant disease in the post-mortem room was strikingly illustrated by analysis of 1000 post mortems at the Middlesex Hospital (Stocks, 1924), when anomalies of the thyroid were found in 13·3 per cent of males and 21·2 per cent of females of 500 persons dying of cancer ; whereas only 2·0 per cent of males and 6·5 per cent of females dying of non-cancerous conditions showed anomalies of the thyroid. Professor Barlow excluded from these anomalies secondary invasion of the thyroid by cancer, and the anomalies most frequently found were simple parenchymatous enlargement and adenomatosis, while calcified nodules, cystic changes, atrophy and fibrosis were frequent. The final result of the survey showed that thyroid anomalies occurred in 18·7 per cent of 500 persons dying of cancer and only in 3·9 per cent of 500 persons dying of conditions other than cancer. Although Stocks (1954, personal communication) has recently cast some doubt on the accuracy of the non-cancerous figures while confirming the cancerous ones, it would require a considerable error of observation to influence greatly the 480 per cent preponderance of thyroid anomalies in the cancerous figures.

The majority of hypo-functioning thyroid glands are small and virtually impossible to detect on palpation and indistinguishable clinically from normally functioning glands, and it is common clinical experience that moderately enlarged thyroids are easily missed even when they are not retrosternal, especially when no symptoms direct attention to that area of the neck. Measurement of radio-iodine clearance and of protein bound iodine are therefore essential steps to establish

dysfunction, and these are now being carried out on a series of patients with known malignant disease, some of whom have shown unexpectedly low clearance and P.B.I. values under the age of 50 without other clinical evidence of hypothyroidism. A further series of post-operative cases of carcinoma of various sites, treated with thyroid and other organic iodine preparations is being observed in the follow-up clinics of this hospital, and in due course it should it be possible to report on the results of these investigations.

## ANIMAL EXPERIMENTS.

The influence of thyroid activity on malignant growths in animals is demonstrated in the review of experimental work quoted here, in which both administration and deprivation have been used to show its important effect on dependent neoplasms.

(1) The incidence of successful takes of grafts of granulosa cell tumours, luteomas and tubular adenomas made into the spleen of mice was noted under varying conditions.

| | Successful "takes." | Per cent. |
|---|---|---|
| On normal diet . . . . . | 16 out of 21 . . . . | 76 |
| Same diet plus 0·2 per cent thyroxine (Miller and Gardner, 1950) | 2 out of 20 . . . . | 10 |

*Comment.*—The presence of excess of thyroxine in the tissues appears to be prejudicial to the successful grafting of tumours from one mouse to another.

(2) The incidence of tumours at the site of injection of chemical carcinogens is reduced by a single injection of thyroxine, and metabolic studies have shown that the rate of disappearance of the carcinogen from mice is significantly increased by thyroxine treatment. Four weeks after injection of 1, 2, 5, 6-dibenzanthracene into mice treated or untreated with thyroxine, the amount of carcinogen remaining in the carcases, estimated spectroscopically, is significantly lower with thyroxine treated animals. Conversely, dibenzanthracene exerts an inhibitory effect on the toxic effect of thyroxine with anoxia survival tissue as the criterion. When the dose response curve of mice to the sarcomagenic action of dibenzanthracene is studied (injections of 5, 16, 48 and 1000 $\mu$g. of DBA) it is found that simultaneous administration of thyroxine significantly lowers the tumour response. This also is true if thyroxine is given at different sites in the animal. Thiouracil feeding markedly increases the incidence of DBA tumours produced by 1 mg. of DBA (Bather, 1952).

(3) Rats bearing Walker Rat Carcinoma 256 were treated with natural thyroxine. Of these 27 per cent showed complete remission and 12 per cent "favourable" histological response, 68 per cent. showing no response at all. Another group treated with synthetic hormone gave only 2 per cent of remissions and no response in 98 per cent of animals. 158 animals were involved (Herbut, Kraemer, and Jacksen, 1950).

*Comment.*—Regression of rat tumours may be induced by thyroxine the natural hormone being superior to the synthetic.

(4) Feeding butter yellow (*p*-dimethylaminobenzene) to rats produced malignant neoplasms of the liver. The addition of wheat, yeast or casein to the diet all delayed the onset of tumours as compared with the controls. (Rusch and Baumann, 1945).

*Comment.*—Wheat, yeast and casein are all proteins with moderately high content of tyrosine, thyroxine being derived by stages from tyrosine and iodine, tyrosine deficiency being a recognised cause of hypothyroidism.

(5) A rat thyroid tumour occurred after prolonged methyl thiouracil administration. This tumour could be transplanted to another rat provided the second rat was first rendered thyroid deficient. In the second " take " rats it underwent anaplastic changes and could then be transplanted into yet other rats without thyroid deficiency. At this stage the tumour had presumably become autonomous after being dependent. (Purves, Griesbach and Kennedy, 1951).

(6) The course of growth of spontaneously occurring mammary tumours in mice was observed. Most had multiple tumours. Many grew conspicuously during pregnancy and regressed after parturition, but recurred promptly when the mouse became pregnant again. Foulds is convinced that the phenomenon is hormonal, but the mechanism remained obscure for apparently it is not due to the action of progesterone or oestrogen. (Foulds, 1949).

*Comment.*—Thyroid deficiency during pregnancy has long been recognised (Marine and Lenhart, 1909 ; Marine, 1935) and might well account for the findings of this experiment.

This review of a selection of experimental work designed to show the action of thyroid in tumour production and regression, contributes further support to the geographical and clinical observations already set out to correlate thyroid deficiency and cancer incidence.

## PHYSIOLOGICAL AND PATHOLOGICAL DATA.

Under normal conditions the thyroid is closely associated with (*a*) Tissue Oxidation, (*b*) Growth, (*c*) Development. These three functions are conveniently compared with conditions that exist in malignant neoplasms.

| | |
|---|---|
| *Tissue oxidation.*<br>Thyroid stimulates metabolism by increasing oxygen consumption, probably by catalysing the enzyme systems which are responsible for tissue oxidation processes. | Malignant tissues, in comparison with normal tissues and benign tumours, are characterised not only by having the lowest concentration of Cytochrome C, but also by having the greatest disparity between the components of the oxidase–cytochrome system. Malignant tissues also show very low amounts of catalase and of the flavin enzymes and co-enzymes (Greenstein, 1947). |
| *Growth.*<br>Thyroid promotes normal growth under normal physiological conditions. | Most carcinogenic substances, in particular the potent hydrocarbons but also those of the urethane group, have been shown to have a considerable growth restraining influence (Haddow, 1938). |
| *Development.*<br>Thyroid brings about development and differentiation of tissue. | Histologically, malignant disease tends to show a dedifferentiation or reversion towards anaplastic tissue pattern that is often compared to embryonic or foetal type of cell. |

## DISCUSSION.

It is instructive to put these observations on a footing with the present knowledge of tumour regression.

First, it may be recalled that it has been variously estimated that spontaneous regression of tumours occurs in man once in about 5000 cases of malignant disease.

This in itself commends to many the idea that cancer has a chemical and probably a hormonal basis, for comparatively sudden changes in the function of endocrine glands either to the plus or to the minus direction is a familiar observation, and in particular with regard to the thyroid.

Secondly, it is relevant to note the comparative frequency of multiple primary malignant neoplasms, for this interesting condition suggests that the body can fairly readily develop a malignant diathesis, and the most likely explanation for this is a hormonal change, the sites of the primary growths being determined by siting factors, which are better understood but which are not within the scope of this present enquiry. A survey by Williamson (1950) of the work of well known English and Continental morbid anatomists gives a figure of 4 per cent for the incidence of multiple primary carcinomata of all autopsies of malignant cases. In view of the comparatively short expectation of life in the average patient with cancer this figure of 4 per cent is probably higher than might at first appear, for the time available to these persons to develop fresh primaries is inevitably short. Furthermore, when it is remembered how easy it is to regard every mass of growth both at the bedside and at autopsy, as an " obvious " metastasis, it is perhaps not altogether surprising that this figure is not higher. Nevertheless, multiple primary neoplasms, even apart from mesenchymal tumours, are common experience to both clinician and morbid anatomist alike, and some would go so far as to think that their numbers are on the increase, or possibly that they are being recognised more freely.

TABLE II.—*Incidence of Multiple Primary Carcinoma.*

(After Williamson, 1950.)

Synchronous double primary carcinomata.

| Author. | Number of necropsies. | Number of malignant cases. | Number of multiple malignancy. |
|---|---|---|---|
| Hanlon | 3000 | 950 | 18 (1·9%) |
| Bilello | 8024 | 1154 | 7 (0·5%) |
| Bugher | 4394 | 983 | 30 (3·1%) |
| Austin | 8124 | 887 | 24 (2·7%) |
| Burke | 2033 | 583 | 46 (7·8%) |
| Tullis | 6836 | 1044 | 21 (2·0%) |
| Warren and Gates | — | 1075 | 40 (3·7%) |
| Warren and Ehrenreich | — | 2829 | 194 (6·8%) |
| Total | — | 9495 | 380 |

Average rate of multiple primary malignancy = 4·0 per cent.

Unfortunately it is not possible in the present state of knowledge to give any accurate account of the effect of hormones on the observed temporary regression of tumours either in man or in animals, for in this field there are too many gaps in our knowledge, too many uncertainties and a certain residuum of conflicting evidence. But the results of adrenalectomy, castration and the administration of oestrogens and sometimes of androgens, all seem to point to a suppression, if only temporary, of some of the functions of the pituitary, at least in man. The recent work of Moon, Simpson, Li and Evans (1950*a*, 1950*b*, 1952*a*, 1952*b*) has convincingly confirmed the conclusions of earlier workers twenty years ago (Ball and Samuels, 1932; Bischoff, Maxwell and Ullmann, 1934), that whereas extracts of the anterior pituitary cause tumours to arise in a variety of sites in laboratory

rodents, extirpation or irradiation of the pituitary will either slow up the rate of established tumour growth or will effectively inhibit the carcinogenic effect of such powerful substances as methylcholanthrene. Recently hypophysectomy by Luft and Olivecrona (1953) in a series of women with advanced mammary carcinoma who had already been treated with every standard surgical hormonal and radiation therapy without effect, appears to have reproduced in man the results observed in animals twenty years ago.

Attempts to bring about tumour regression by castration or adrenalectomy are, for the most part, merely applications of those animal experiments, but acting through their indirect effect on the pituitary. The administration of oestrogens aims at bringing about a partial, if temporary, medical hypophysectomy, though unfortunately oestrogens and androgens have been shown to possess moderately active carcinogenic properties (though not the synthetic stilboestrol), and that oestrogens have in addition a noticeable mitogenic effect on sensitive tissues (Bullough, 1942 1946, 1950*a*, 1950*b*). The final drawback to oestrogen therapy, apart from its minor side effects, is that the pituitary, after a period of medication, becomes indifferent to the action of stilboestrol. Indeed, it was shown by Burroughs and Horning (1947) that if the pituitary is given a prolonged treatment with oestrogens the anterior lobe as a whole passes into a stage of hyperplasia having all the characters of an adenoma.

In common with other endocrine glands, the thyroid has a reciprocal relationship with the pituitary. Thus, the gonadotrophic hormone from the pituitary will stimulate the ovary to produce oestrogen and the blood level of oestrogen so produced in turn governs the subsequent output of gonadotrophic hormone, a low oestrogen level leading to a high output of gonadotrophic hormone and *vice-versa*, a high oestrogen blood level leading to a low gonadotrophic hormone output by the pituitary. In the same way, a low thyroxine level in the blood will act as a stimulus to the pituitary which will put out increased amount of thyrotropic hormone to stimulate, in its turn, the thyroid ; and conversely, a high thyroxine level in the blood has the effect of suppressing the activity of the pituitary. Thus both oestrogen and thyroxine have a similar action on the pituitary and tend to suppress its activity, but in addition thyroxine has not been shown to possess any carcinogenic properties, nor would such activity be expected from consideration of its molecular structure ; nor has it been shown that the pituitary eventually becomes indifferent to its action. Furthermore, the presence of thyroxine in the tissues, as has already been amply demonstrated in the animal experiments detailed above, brings about a tissue environment that is unfavourable to tumour growth and development, at least as long as tumours remain in the dependent phase.

In an attempt to explain how this change in tissue is effective we are left with several possibilities :

(*a*) That thyroxine encourages normal physiological tissue respiration rather than the so-called anaerobic type which appears to be the one demonstrable biochemical difference between normal and neoplastic tissue (Greenstein, 1947).

(*b*) That thyroxine in adequate amounts may reverse the process of dedifferentiation or drift towards anaplastic growth that characterises most neoplastic tissue—the more malignant, the more anaplastic—that is, it may bring about the process of differentiation and development that it certainly does promote in the embryonic stages and early years of life.

(*c*) That thyroxine, by raising the metabolic rate or by maintaining it at a

proper level, brings about some degree of katabolism and/or an increased rate of excretion of the carcinogen.

(*d*) That thyroxine brings about some depression of pituitary activity. In this connection, the depolymerisation of connective tissue which seems to be brought about by excess of thyrotropic hormone (Robb-Smith, 1954) is particularly relevant in view of the importance which some attach to changes in connective tissue in the genesis of malignant disease, for thyroid medication is capable of reversing the process and of bringing about polymerisation of the mucopolysaccharides and so a return to a more normal structure of connective tissue.

(*e*) That the favourable effect of thyroxine in these experiments is due to a combination of some or all of these factors.

At a recent Imperial Cancer Research Fund lecture at the Royal College of Surgeons, Professor Hadfield said : "It is my firm belief that for many years we have been so anxious to discover all we possibly can about the structure of the growth and its metastases that we have almost forgotten the soil in which it grows." (Hadfield, 1954.)

In coming to any conclusion regarding the possible association between malignant disease and thyroid function, it cannot be too strongly stressed that a low metabolic rate or an insufficiency of thyroid substance can in no way be considered as a primary cause of cancer. Thyroid and related substances cannot therefore be considered as a cure for cancer. It is, however, suggested that thyroid function (or dysfunction) may be associated with the susceptibility or immunity to cancer. As such, thyroid might well be used as a therapeutic weapon, ancillary only to accepted surgical treatment, much as antiserum is a valuable ancillary in the treatment of tetanus or gas gangrene, but in itself is no substitute for radical surgery in either condition, even though it may rightly be regarded as the factor which takes suitable surgical toilet out of the realm of debatable value to the sphere of accepted success under reasonable conditions.

In the wider and perhaps more important field of preventive medicine, the possibility that an increased susceptibility to cancer occurs in those with a poor thyroid function leads for the first time to a real chance of adopting prophylactic measures against cancer on a wide scale. The measures adopted would be in the main those already available against goitre in the young and in adolescents, with the idea of building up healthy active thyroid glands during their period of development ; and in adult life steps would be taken to maintain a good level of thyroid activity by ensuring that iodine is available in suitable quantities in food and drink, especially when middle life is reached and there is a natural tendency for the iodine level to fall (Rundle, 1951). Only in some such way can an immunity be built up against the many carcinogenic substances which it is virtually impossible to avoid, largely due to the widespread use of coal and oil. In addition, it must be recalled that there are substances such as cholesterol, vitamin D and the sex hormones and even sunshine itself, which are all part of a normal and healthy existence in spite of their proved capacities to cause cancer, even if they are not finally shown to be the primary causes of the commonly occurring, non-industrial neoplasms that form the bulk of the problem of malignant disease.

SUMMARY.

Data from fifteen countries in four continents give support to the importance of local factors to account for the known local variations of cancer incidence.

Iodine availability, traced by goitre incidence, appears to be one of such factors. Closer scrutiny of some of these countries corroborates these conclusions.

Clinical findings and a review of some of the experimental work available lend further emphasis to these observations.

The apparent relationship of thyroid insufficiency and the liability to develop cancer is discussed in connection with such other hormonal influences over cancer as are already known, particularly in respect of the pituitary.

Lines of investigation and clinical trials have been started, but results of any value cannot be expected for several years.

A non-specific organ immunity or susceptibility seems to be the simplest explanation of the facts presented, and the possibilities, preventive and perhaps therapeutic, that are opened up by this line of research are briefly discussed.

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## REFERENCES.

Ball, H. A., and Samuels, D. T.—(1932) *Amer. J. Cancer*, **16**, 351.
Bather, R.—(1952) *Cancer Res.*, **12**, 247.
Bayard, O.—(1919) ' Beitrage zur Schilddrusenfrage ', Basel (Schwabe), p. 42.
Bishoff, F., Maxwell, L. C., and Ullmann, H. J.—(1934) *Amer. J. Cancer*, **21**, 329.
Bullough, W. S.—(1942) *J. Endocrin.*, **3**, 150.—(1946) *Philos. Trans.*, B, **231**, 453.—(1950*a*) *J. Endocrin.* **6**, 340.—(1950*b*) *Ibid.*, **6**, 350.
Burrows, H., and Horning, E. S.—(1947) *Brit. med. Bull.* **4**, 367.
Campbell, J. M. H.—(1925) *Quart. J. Med.*, **18**, 191.—(1927) *J. Hyg., Camb.*, **26**, 1.
Chu, J. P., and You, S. S.—(1945) *J. Endocrin.* **4**, 115.
Clinquart, E.—(1926) *Bull. Acad. Méd. Belg.*, **6**, 505.
Foulds, L.—(1949) *Brit. J. Cancer*, **3**, 345.
Greenstein, P.—(1947) ' Biochemistry of Cancer '. New York (Academic Press).
Haddow, A. J.—(1938) *J. Path. Bact.*, **47**, 567.
Hadfield, G.—(1954) *Ann. Roy. Coll. Surg.*, **14**, 21.
Herbut, P. A., Kraemer, W. H. and Jacksen, J.—(1950) *Cancer Res.*, **10**, 224.
Hoffman, F. L.—(1915) ' The Mortality from Cancer throughout the World.' Newark (Prudential Press), pp. 122, 128.
Joslin, E. P.—(1946) ' The Treatment of Diabetes mellitus,' 8th ed. London (Kimpton), pp. 262, 653.
Kelly, F. C.—(1946) ' World Goitre Survey.' London (Iodine Educational Bureau).
Kolmer, J. A.—(1949) Laboratory Examinations, 2nd ed. New York (Appleton Century), p. 177.
Legon, C. D.—(1951) *Brit. J. Cancer*, **5**, 175.—(1952) *Brit. med. J.*, ii, 700.
Luft, R., and Olivecrona, H.—(1953) *J. Neurosurg.* **10**, 301.
Machle, W., and Gregorius, F. (1948) *Congr. int. Mal. prof.*, **9**, 463.

MARINE, D.—(1935) *J. Amer. med. Ass.*, **104**, 2334.
*Idem* AND LENHART, C. H.—(1909) *Arch. intern. Med.*, **4**, 440.
MCCLENDON, J. F.—(1939) 'Iodine and the Incidence of Goiter.' Minneapolis (University of Minnesota Press.)
MCEWAN, P.—(1938) *Brit. med. J.*, i, 1037.—(1948) 'The Clinical Picture of Thyrotoxicosis.' Edinburgh (Oliver & Boyd).
MILLER, O. J., AND GARDNER, W. U.—(1950) *Cancer Res.*, **10**, 233.
MOON, H. D., SIMPSON, M. E., LI, C. H., AND EVANS, H. M.—(1950*a*) *Cancer Res.*, **10**, 297.—(1950*b*) *Ibid.*, **10**, 549.—(1952*a*) *Ibid.*, **12**, 448.—(1952*b*) *Science*, **116**, 331.
MURRAY, M. M., RYLE, J. A., SIMPSON, B. W., AND WILSON, D. C.—(1948) M.R.C., Memorandum 18. London (H.M.S.O.).
NORDLING, C. O.—(1953) *Brit. J. Cancer*, **7**, 68.
PURVES, H. D., GRIESBACH, W. E., AND KENNEDY, T. H.—(1951) *Brit. J. Cancer*, **5**, 301.
ROBB-SMITH, A. H. L.—(1954) 'Lectures on General Pathology,' p. 567. London. (Lloyd-Luke).
RUNDLE, F. F.—(1951) 'Joll's Diseases of the Thyroid.' London (Heinemann), p. 78.
RUSCH, H. P., AND BAUMANN, C. A.—(1945) Research Conference on Cancer, Washington.
SOURASKY, M.—(1928) 'International London Conference on Cancer.' Bristol (John Wright), p. 536.
STOCKS, P.—(1924) *Biometrika*, **16**, 364.—(1925) *Ibid.*, **17**, 159.—(1928) 'International London Conference on Cancer.' Bristol (John Wright), p. 508.—(1947) 'Studies on Medical Population Subjects.' No. 1. London (H.M.S.O.).—(1950) *Brit. J. Cancer*, **4**, 147.—(1953) *Brit. med. J.*, ii, 847.
UPDEGRAFF, H. L.—(1933) *J. Amer. med. Ass.*, **101**, 1138.
WAYNE, E. J.—(1954) *Brit. med. J.*, i, 411.
WILLIAMSON, T. B.—(1950) *Ibid.*, i, 648.
WINSBURY-WHITE, H. P.—(1948) 'Textbook of Genito-Urinary Surgery.' Edinburgh (Livingstone), p. 525.
World Health Organisation. (1952*a*) *Epidem. vit. Stat. Rep.*, **5**, 1.—(1952*b*) *Ibid.*, **5**, 371.

# THE MORBID ANATOMY OF CARCINOMA OF THE BRONCHUS: AN ANALYSIS OF 87 CASES, WITH SPECIAL REFERENCE TO SOLITARY CEREBRAL METASTASES.

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ALTHOUGH a study of the morbid anatomy of carcinoma of the bronchus can have little bearing on the important problem of etiology, nevertheless any ascertainable facts about this increasingly common disease may contribute something to our knowledge of it.

Recent comprehensive surveys of the pathology of carcinoma of the bronchus have been made in this country by Harrison (1950), on 353 confirmed cases seen at St. Thomas's Hospital during a 13 year period, and by Bryson and Spencer (1951), who made a clinical and pathological study of 866 cases, based on material gathered from 26 London County Council hospitals in the greater London area.

The cases here presented all came to autopsy in one provincial hospital during the ten year period, 1943–1952, and although their number is small by comparison with the two London series, it seemed worth while to analyse them especially from the point of view of cerebral metastases, to which little attention has hitherto been directed. (To be strictly accurate, three autopsies were actually performed at St. Crispin Mental Hospital to which the patients had been removed on account of mental symptoms shortly before death, and one at Danetre Hospital, Daventry). The brain was available for examination in 55 of the 87 cases. Figures for cerebral metastatic deposits are therefore based on this number.

Those autopsies not personally performed were, with few exceptions, witnessed by me.

The histology of 41 cases accessible to personal study is included for comparison with the types of cell structure given in other series.

*Frequency of carcinoma of the bronchus.*

During the ten year period under review the total number of autopsies performed in Northampton General Hospital was 3483. The post mortem diagnosis of carcinoma of the bronchus in 87 cases reveals this disease as the cause of death in 2·4 per cent of all verified cases, a not inconsiderable proportion.

*Sex and age incidence.*

The figures of the sex incidence approximated to those generally accepted in this country. Of the 87 cases, 73 (84·0 per cent) occurred in males, 14 (16·0 per cent) in females, the ratio of males to females being just over 6 : 1. Recent Norwegian figures quoted by Kreyberg (1952) for 122 autopsies carried out by Jakobsen gave the sex ratio rather surprisingly as only 2 : 1 although in 100 clinical cases the ratio was 5 : 1.

Likewise the age incidence agreed with that previously reported. Of the 87 cases, 54 fell into the age group 51–70 years, there being as many (27) in the age group 51–60 years as in the group 61–70 years. The average age at which death occurred was 56·4 years, slightly higher in males (56·7 years) than in females (54·7 years). The average age at death was much lower than that in Bryson and Spencer's (1951) series (59·5 years), but almost identical with that given by Harrison (1950) for his confirmed cases (56·6).

*Ages at time of death in decades.*

| Ages | 31–40 | 41–50 | 51–60 | 61–70 | 71–80 | 81–90 |
|---|---|---|---|---|---|---|
| Cases | 4 | 24 | 27 | 27 | 4 | 1 |

*Geographical distribution.*

This hospital receives patients from the surrounding rural areas as well as from the borough. Of the 87 patients 73 (84 per cent) had lived in the town, 14 (16 per cent) in rural districts.

*Distribution of growths in the lungs*

The tumours originated on the right side in 49 cases and on the left side in 38. These figures correspond to those of Willis (1952) who in his 84 autopsy cases found that the right side was involved in 45 cases, the left in 36. In Harrison's (1950) 353 confirmed cases the right side was also more commonly affected than the left (59·2 per cent of tumours had their origin on the right side, 39·4 per cent on the left.) Bryson and Spencer (1951) on the other hand did not find any difference in the site of the origin on the two sides.

In the present series there were 6 tumours in the periphery of the lung with no apparent bronchial origin. Of these, 2 were apical growths, both on the right side. In 2 of the cases with peripheral tumours, 1 apical, the other in the left upper lobe, the only metastases were solitary ones in the brain. In 3 cases the growth was so extensive, involving the whole of the lung substance, that no origin was ascertainable.

The distribution of growths in the bronchi was as follows :

| | Right side. | | Left side. |
|---|---|---|---|
| Main brochus | 18 | Main bronchus | 7 |
| Bronchus to right upper lobe | 7 | Bronchus to left upper lobe | 9 |
| Bronchus to right lower lobe | 11 | Bronchus to left lower lobe | 12 |
| Bronchus to right middle lobe | 1 | | |

Excluding the 6 peripheral tumours and the 3 cases of massive growths there were 13 cases in which no description of the distribution of the tumour other than " right or left side " was available.

In view of the small number of cases it would be unwise to state that the right main bronchus appears to be the commonest site of origin of bronchial carcinoma. All that can be said is that in this series the tumour originated most often in that site, especially as Harrison (1950) in his series of 353 cases found that the right main bronchus was only slightly commoner than the bronchi to the right upper and lower lobes as the site of origin. It is also significant that Sellors (1953) in his series of 200 clinical cases found that the carcinoma originated in 50 cases

in the bronchus to the left upper lobe and in 43 cases in the bronchus to the left lower lobe; the site of origin was the bronchus to the right upper lobe in only 3 cases.

*Metastatic deposits.*

Thoracic and abdominal metastases were present in 68 of the 87 cases (78·1 per cent). In addition, cerebral metastases without any visceral deposits occurred in 7 cases. Therefore the true metastatic figure was 75 (86·2 per cent), a much higher rate of metastasis than was given by Bryson and Spencer (1951), who in their survey of 866 cases gave the figure of secondary deposits as 72·3 per cent. It is possible that the figure in the present series would have been even higher had the brain been examined in all cases, but as has already been mentioned, the skull was only opened in 55 of the 87 cases and the figures for cerebral deposits are therefore based on this number.

*Visceral metastases.*

The organs most commonly invaded by secondary deposits were as follows:

| | | Per cent. | | | Per cent. |
|---|---|---|---|---|---|
| Liver | 33 | (37·9) | Adrenals | 12 | (13·7) |
| Abdominal glands | 23 | (26·4) | Pericardium | 11 | (12·6) |
| Bones | 23 | (26·4) | Pancreas | 10 | (11·4) |
| Ribs | 14 | | Spleen | 5 | ( 5·7) |
| Vertebrae | 9 | | Kidney | 3 | ( 3·4) |
| Opposite lung | 15 | (17·2) | | | |

Metastases were present in the thyroid in 2 cases and in the jejunum and aorta in one case each.

Skeletal deposits were more frequent in this series (26·4 per cent) than in that of Bryson and Spencer (1951), who found them in only 13 per cent, but Willis (1952) observed bony metastases in about 25 per cent of his cases. The other discrepancy between the present series and that of Bryson and Spencer is the higher proportion in this series of secondary deposits in the pancreas (11·4 per cent as against 3·6 per cent). On the other hand both Willis and Bryson and Spencer found metastases in the adrenals much more often than they were discovered in this series. (Willis 40 per cent, Bryson and Spencer 23 per cent, present series 13·7 per cent). These differences cannot be explained; they can only be recorded.

*Cerebral metastases.*

Out of the 55 brains examined, cerebral metastases were present in 14 (25·4 per cent). The frequency with which cerebral deposits have been recorded in carcinoma of the bronchus has varied widely with different observers. Those who have investigated metastatic tumours in the brain have found that a very high proportion of these had their primary source in the lung. Elkington (1935), who analysed the records of the National Hospital for the period 1918–1933, found that of 72 metastatic cerebral tumours, 24 (33 per cent) originated in the bronchus. He also recorded 17 cases of which 9 (52 per cent) were of bronchial origin. Ferguson and Rees (1930), from the same Hospital, described 9 primary bronchial tumours with secondary cerebral deposits, collected over a 10 year period. Globus and

Meltzer (1942), who performed 33 complete autopsies on patients who had secondary growths in the brain traced the primary growth to the lung in 19 cases, the very high proportion of 57·5 per cent. Conversely, those who have studied the morbid anatomy of carcinoma of the bronchus with only an incidental interest in cerebral metastases have, in general, found that cerebral metastases from this source occur in a quarter to a third of the cases. The highest recorded figure is that of Fried and Buckley (1930), who recognized secondary cerebral deposits in 41 per cent. Other observers have given much lower figures: Reingold, Ottoman and Konwaler (1950) 30·5 per cent, Willis (1952) 21 per cent, Bonser (1934) 16 per cent, Harrison (1950) 20 per cent, Bryson and Spencer (1951) 17 per cent, Simpson (1929) 13·5 per cent (in a series of 139 cases), Kilkuth (1925) 12·6 per cent (in a series of 246 cases).

Few authors have differentiated between multiple cerebral deposits from carcinoma of the bronchus and large single masses except to say that the former are common and the latter rare, though Kilkuth (1925) reported single masses in 13 of 31 cases with cerebral metastases.

Although the present series is small it is noteworthy that in 14 cases with cerebral metastases only 6 were multiple and 8 single. The multiple deposits were widely distributed and showed no predilection for any particular area of the brain. In all except one of the cases with multiple cerebral metastases there were also secondary deposits in the viscera. On the other hand, of the 8 cases with single metastases only two had visceral deposits. In other words, there were 6 cases in which the sole metastasis was in the brain. Brief notes on these cases are appended. All details not strictly relevant have been omitted.

*Case* 1.—G. S—, a man aged 49 years, was admitted to hospital 22.xii.51 from a sanatorium, where he had been treated for tuberculosis for 4 months. A fortnight prior to admission to hospital he had developed a left hemiplegia and had since remained semi-comatose and incontinent. On examination there was a complete hemiplegia and a dubious hemianopia. Severe neck rigidity was present, but examination of the cerebro-spinal fluid revealed a normal fluid except for pressure, which was 190 mm. The only abnormality disclosed by examination of the chest was diminished air entry in the upper zone of the left lung with deviation of the trachea to the left. The differential diagnosis made was between tuberculous encephalitis and cerebral thrombosis. Death took place 2 days after admission.

At post mortem dense fibrous adhesions were found almost completely to obliterate the left pleural cavity. There were old fibrous adhesions at the right apex. The trachea and main bronchi contained a considerable amount of muco-pus. Arising from, and producing almost complete obstruction of the left upper lobe bronchus was a hard, white tumour, approximately 5 cm. across. Distal to this tumour the lobe was collapsed and contained a number of irregular cavities filled with thick yellow pus. The left lower lobe was studded with a number of nodules of tumour up to 0·5 cm. across. In the upper half of the right upper lobe there was an irregular tuberculous focus 2 cm. across consisting of a central caseous mass enclosed in dense fibrous tissue. The left hilar lymph nodes were replaced by tumour. No abnormalities were found in the abdomen. The skull and meninges were normal. The cerebral convolutions were flattened. In the right hemisphere between the parietal cortex and the internal capsule, which was displaced medially, there was a large spherical metastasis with a necrotic centre. No other metastasis was found.

Histologically the tumour was a pure squamous-celled carcinoma.

*Case* 2.—A. M—, a married woman aged 62 years, was admitted 31.v.49 in a drowsy state, unable to give a good history. Her main complaint was loss of weight and a cough of 3 months' duration, which had been accompanied by a blood-stained sputum. On examintion the fundi could not be seen. The right pupil was sluggish and the tongue was protruded to the right. The reflexes were normal. The cerebro-spinal fluid was normal except for a

raised pressure of 160 mm. and a raised protein of 53 mg. per cent. An X-ray showed a mass extending from the left hilum and involving the mid and lower portions of the left upper lobe. The clinical diagnosis was carcinoma of the lung with cerebral metastases. The patient died 12 days after admission.

At post mortem a large, white, firm tumour mass was found involving the whole of the medial portion of the upper lobe of the left lung. No mediastinal glands were infiltrated and no other deposits were found in the viscera. The skull was normal, but attached to the falx cerebri at the vertex of the brain, and pressing on the pre-Rolandic portion of the left superior frontal gyrus there was a firm hard tumour the size of a cherry (proved histologically to be a meningioma). The cerebral convolutions were somewhat flattened. On section of the brain a soft, reddish tumour was found in the tegmental portion of the pons, occupying the whole of the formatio reticularis and medial longitudinal bundle and occluding the fourth ventricle. The tumour extended upwards, involving the red nucleus, the tegmentum, both lemnisci and the iter. The extreme caudal end of the thalamus was also infiltrated by tumour. Histologically the tumour was pleomorphic, consisting of oat cells, spindle cells and small polygonal cells.

*Case* 3.—R. S—, a married woman aged 54 years, was admitted to hospital 26.xii.47. She had been successfully treated with radium in 1942 for carcinoma of the cervix and had had no recurrence. Her complaint was of headaches and vomiting for 3 weeks. On immediate examination a weakness of the left face was noted and there was slow nystagmus on lateral and upward deviation of the eyes. The reflexes were all exaggerated but the plantar responses were flexor. A diagnosis of cerebral tumour was made tentatively, but before further investigations could be carried out the patient died suddenly the day after admission.

At post mortem a fungating, cauliflower-like growth was found in the upper lobe of the left lung. No enlarged mediastinal glands were present. The pelvis showed no evidence of any local or secondary lymphatic involvement from the cervix. The brain was moderately congested and oedematous. The left half of the cerebellum was replaced by a degenerating, cystic tumour.

Histologically the growth in the lung and the secondary deposit in the cerebellum were found to be an unspecified type of carcinoma of the bronchus. (Sections were not available for personal study.)

*Case* 4.—A. T—, a man aged 62 years, was admitted 30.iii.48 with a history of " chronic bronchitis " and convulsive attacks with loss of consciousness which began a fortnight before admission. Six months previously, when he had attended hospital as an out-patient, an X-ray had been suggestive of bronchial neoplasm, with displacement of the mediastinum to the right side. On the day of admission he had another convulsive attack and thereafter remained semi-conscious until death, which occurred 3 days later. On examination there was a left flaccid hemiplegia. The pupils were normal. The left fundus was blurred ; the right was not seen. The clinical diagnosis was carcinoma of the bronchus with secondary cerebral deposits.

At post mortem the right lung was found to be firmly adherent to the posterior thoracic wall from the hilum to the mid-axillary line. Below this the pleural cavity contained many dense fibrous adhesions. The bronchus to the lower lobe was almost completely blocked by soft, creamy tumour. Lateral to this the lung field contained an apparently circumscribed spherical nodule of growth, 4 cm. across. In the surrounding lung there were small islands of a similar nature. Distal to the obstruction the bronchi were grossly distended, with thinned reddened mucosa, and filled with thick, yellow pus. The middle and upper lobes were solid, dark red, and studded with confluent greyish yellow areas from which pus could be expressed. No abnormalities in the abdomen were detected. In the brain at the summit of the vertex of the right cerebral hemisphere there was a rounded metastasis, 3 cm. across, consisting of a central cavity lined by a thin lamina of cream-coloured tumour.

Histologically the tumour was composed mainly of columns of large adenocarcinoma cells, but a few oat cells were interspersed between the columns.

*Case* 5.—H. T—, a man aged 53 years, was admitted to hospital 16.i.48. with a history (obtained from his wife) of abdominal pain and vomiting for 3 months, following an attack of " gastritis." Since this attack he had become more and more apathetic and absent-minded and had had some unsteadiness in walking. Since December, 1947, he had been confined

to bed, unable to walk. There had been much loss of weight. For a week prior to admission he had been completely incontinent of urine and faeces. On examination there was evidence of collapse at the base of the left lung. The fundi and cranial nerves were normal. In the arms there was a coarse, regular tremor, especially on movement, with much increased tone. The reflexes were exaggerated. In the legs tone was also increased and the reflexes were exaggerated, particularly on the left side. The left plantar response was extensor. The cerebro-spinal fluid was normal apart from an increased pressure of 250 mm. An X-ray of the chest gave evidence of neoplastic formation in the left hilar region with associated collapse at that base. Death occurred 3 days after admission.

At post mortem the left lung was found to be collapsed and the left pleural cavity contained about 500 c.c. of golden yellow fluid. The left main bronchus was compressed by a soft, buff-coloured tumour, about 8 cm. in diameter, which was covered by a layer of lung 2 cm. thick. This layer of lung was relatively airless and thick yellow pus was expressed from the cut surface. The oesophagus and aorta were displaced posteriorly by the tumour. No abnormalities were found in the abdomen other than congested liver and nodular cortical hyperplasia of both adrenals. In the brain there was flattening of the cerebral convolutions. The lateral ventricles were slightly dilated; the third ventricle rather widely dilated. The upper half of the midbrain was occupied by a soft, yellowish tumour which did not extend into the pons. Histologically this tumour was composed entirely of small "oat cells."

*Case* 6.—A. V—, a man aged 46 years, was admitted to hospital 9.ix.46 on account of frontal headaches, throbbing in character and especially severe in the mornings, which had been present for 3 weeks. Just before admission much nausea and vomiting had developed. There had been no cough at any time. On examination papilloedema was present on both sides. The pupils were equal and reacted to light and to accommodation. The arm and leg reflexes were normal, but some past pointing was noted in the right arm. Lumbar puncture gave a yellow fluid under 300 mm. of pressure. The protein was increased to 120 mg. per cent but otherwise the fluid was normal. An X-ray of the chest was suggestive of a growth in the hilum of the right lung. The patient deteriorated rapidly and died 3 weeks after admission.

At post mortem a hard, white tumour, 1 in. in diameter, was present at the apex of the right lung. A mass of infiltrated glands was present on the right side of the trachea, extending upwards into the neck, above the right clavicle. The bifurcation gland was also enlarged and replaced by tumour. No abnormalities were found in the abdomen. In the brain there was a secondary deposit, just over 1 in. in diameter, deep in the left temporo-parietal lobe. The mass impinged on the left lateral ventricle, which was slightly dilated. The brain tissue surrounding the tumour was soft and oedematous, and the tumour was easily detached from the adjacent cerebral substance. Histologically the tumour was a small oat-celled carcinoma. The other 2 cases in which a single cerebral metastasis occurred, one in the lateral border of the right cerebellar hemisphere, the other in the right lateral lobe of the cerebellum, also had visceral deposits, and are therefore not presented in detail.

## COMMENT.

Of the 6 cases in which solitary cerebral metastases were present, 4 were diagnosed clinically, although the metastases were considered to be multiple, not single; one case was correctly diagnosed as a cerebral tumour although death occurred before investigations were completed and in only one case was the correct diagnosis not established before death. All 6 cases had neurological symptoms, but in only one (Case 3) were these of localizing value. The solitary cerebral metastases did not affect any particular regions of the brain, though perhaps it is worthy of note that none was present in the cerebral cortex posterior to the parietal lobe.

The real interest of these cases of solitary cerebral deposits lies in the fact that they were "solitary", i.e., they were the only metastatic deposits in the body. From the histology (2 were of the small oat cell type and 2 others contained

oat cells), a wide dissemination of the tumour might have been expected, as these forms metastasise early and extensively. No common factor could be found to account for this limitation of secondary deposits to one mass in the brain, but as it occurred in well over a third of the cases in which cerebral metastases were present, it is difficult to accept the finding as one of pure chance. Single massive deposits in the brain have often been recorded, though they have hitherto been considered much less common than multiple ones, but solitary cerebral deposits have as yet received little attention in the literature of carcinoma of the bronchus.

*Histology.*

In the 41 sections accessible to personal examination the small oat cell type of tumour predominated, accounting for 12 cases (29·2 per cent). A mixed type of tumour was the next commonest, occurring in 10 cases (24·4 per cent). The types of cells encountered in these tumours were: alveolar cells and oat cells, 4 cases; alveolar cells and polygonal cells, 2 cases; and one case each of spheroidal cells and squamous cells; polygonal cells and oat cells; spheroidal cells and squamous cells. One case was pleomorphic, the tumour containing oat cells, spindle cells and polygonal cells. There were 9 cases of adenocarcinoma (21·9 per cent). Six cases (14·6 per cent) were classified as anaplastic, the cells being undifferentiated large or small polygonal cells, whilst there were only 4 cases (9·7 per cent) of squamous-celled carcinoma.

Many studies have been made of the histology of bronchial carcinoma and in general the oat celled variety has been found, at least in this country, to be the commonest cell type. Harrison (1950), for example, in a series of 329 sections, listed 48·5 per cent as oat-celled tumours, 26·4 per cent as squamous carcinomas and 25·2 per cent as adenocarcinomas. The proportion of squamous-celled carcinomas was higher than in many series, though not so high as in the Norwegian series studied by Kreyberg (1952), who in 100 sections derived from surgical material found that squamous-celled carcinoma accounted for 56 per cent, oat-celled carcinoma only 21 per cent, "large celled" carcinoma (presumably corresponding to the anaplastic type) 10 per cent and adenocarcinoma only 9 per cent. Bryson and Spencer (1951), on the other hand, only found true squamous celled carcinomas in 6·9 per cent of their series, the commonest cell type being the polygonal cell, which accounted for 40 per cent and the oat cell, 36 per cent. Adenocarcinoma was relatively rare, only 4·9 per cent, while a variety which they described as "squamoid," i.e., resembling the squamous-celled type but not showing cell nests or keratinization, occurred in 11·2 per cent of their series.

By far the largest contribution to the histology of bronchial carcinoma has recently been made by workers at the Mayo Clinic, who in a series of 4 papers have reported on the examination of 849 cases (Carlisle, McDonald and Harrington, 1951; McBurney, McDonald and Clagett, 1951; Patton, McDonald and Moersch, 1951*a*, 1951*b*). There was a striking difference in the frequency of the cell-types described by these authors and those reported by other observers. In the American series only 8·8 per cent were of the oat-celled variety, and adenocarcinoma was also relatively rare (13·2 per cent). The cell-type most often encountered (40·2 per cent) was what the authors designated as "large cell" carcinoma, which, from its description would appear to be classified as "anaplastic" in this country. Squamous-celled carcinoma was also common, (37 per cent) though the percentage was not as high as in Kreyberg's (1952) series (56 per cent) or in that of Reingold,

Ottoman and Konwaler (1950) (66·7 per cent), who studied material from 60 autopsies. It is of interest that only Reingold, *et al* (1950) have referred to the many tumours in which pleomorphism is a characteristic and in which no one type of cell predominates. Such tumours formed nearly 25 per cent of my admittedly small series, and Willis (1952) found that a high proportion of his 84 cases contained at least two, and sometimes three, types of cell.

SUMMARY.

The post-mortem findings in 87 cases of primary bronchial carcinoma have been described.

The age and sex incidence have been discussed.

An account has been given of the cerebral metastases found in 14 of the 55 cases in which examination of the brain could be undertaken. Single secondary deposits in the brain were more common than multiple ones.

Details have been given of the solitary cerebral metastases (i.e., single masses in the brain unaccompanied by visceral deposits) which were present in over a third of the cases with secondary cerebral deposits.

The histology of 41 cases has been briefly reported

It is a pleasure to acknowledge the courtesy of my clinical colleagues for the use of their records.

My thanks are also expressed to Dr. R. M. Heggie, and to Dr. W. E. Bryan, for access to notes of autopsies performed by them.

Since this paper was written, Meyer and Reah from the London Hospital (1953) have recorded 117 cases of secondary cerebral deposits from primary bronchial carcinoma. The metastases were single in 30 per cent of the total number, but in only 2 cases of the 117 were these solitary cerebral deposits the only metastases found in the body.

## REFERENCES.

Bonser, G. A.—(1934) *J. Path. Bact.*, **38**, 209.
Bryson, C. C., and Spencer, H.—(1951) *Quart. J. Med.*, **44**, 173.
Carlisle, J. C., McDonald, J. R., and Harrington, S. W.—(1951) *J. thorac. Surg.*, **22**, 74.
Elkington, J. St. C.—(1935) *Proc. Roy. Soc. Med.*, **28**, 1080.
Ferguson, F. R., and Rees, W. E.—(1930) *Lancet*, i, 738.
Fried, B. M., and Buckley, R. C.—(1930) *Arch. Path.*, **9**, 483.
Globus, J. H., and Meltzer, T.—(1942) *Arch. Neurol. Psychiat.*, **48**, 163.
Harrison, A.—(1950) *St. Thom. Hosp. Rep.*, **6**, 183.
Kilkuth, W.—(1925) *Virchows Arch.*, **255**, 107.
Kreyberg, L.—(1952) *Brit. J. Cancer*, **6**, 112.
McBurney, R. P., McDonald, J. R., and Clagett, O. T.—(1951) *J. thorac. Surg.*, **22**, 62.
Meyer, P. C., and Reah, T. G.—(1953) *Brit. J. Cancer*, **7**, 438.
Patton, M. M., McDonald, J. R., and Moersch, H. J.—(1951*a*) *J. thorac. Surg.*, **22**, 83.
—(1951*b*) *Ibid.*, **22**, 88.
Reingold, I. M., Ottoman, R. E., and Konwaler, B. E.—(1950) *Amer. J. clin. Path.*, **20**, 515.
Sellors, T. Holmes.—(1953) *Proc. Roy. Soc. Med.*, **46**, 861.
Simpson, S. L.—(1929) *Quart. J. Med.*, **22**, 413.
Willis, R. A.—(1952) "The Pathology of Tumours." London (Butterworth).

# ADENOCARCINOMA CELLS IN THE SPUTUM.

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THE following two cases, one of primary adenocarcinoma in the lung, and one probably of pulmonary metastasis from adenocarcinoma of the colon, are considered worth recording as they help to complete the outline given in the communication published in the last edition of this journal (Philps, 1954).

It was then stated that no photograph was available of carcinoma cells from the sputum of a patient subsequently diagnosed histologically as suffering from pulmonary adenocarcinoma. Since that paper was written, two cases have been confirmed.

## *Case 1.*

This patient was mentioned in Table III of the previous paper. His sputum was first found to contain carcinoma cells in February, 1953. He was considered too old for successful operative treatment so he was kept under observation and in February, 1954, was readmitted to hospital with evidence of bone metastasis. He died recently and at post mortem (Dr. V. Udell) an adenocarcinoma was found in the lower lobe of the right lung. There were numerous bone metastases. No other possible primary site was found.

The sputum contained numerous small fragments of tissue composed in many cases of cells with grossly vacuolated cytoplasm (Fig. 1 and 2). Some clumps showed less vacuolation, but marked nuclear pleomorphism (Fig. 3).

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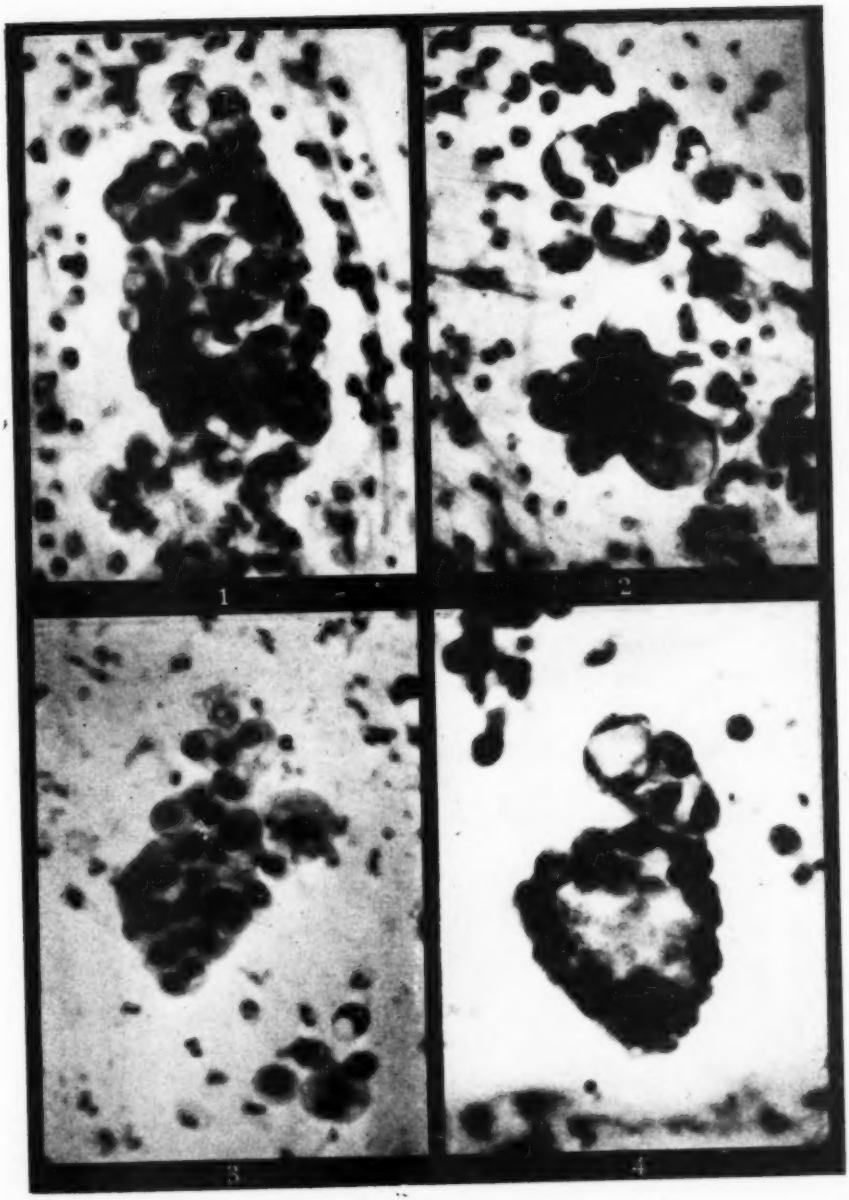
### DESCRIPTION OF PLATES.

*Fig.* 1.—Part of a clump of cells from the sputum of Case 1. Many of the cells show vacuolation of the cytoplasm which displaces the nucleus to one side. Apart from this, there is little irregularity of nuclear shape, size or staining density.
These cells clearly form part of a fragment of tissue in the sputum, and because of the vacuolation of the cytoplasm, were considered to be diagnostic of adenocarcinoma. × 400.

*Fig.* 2.—Another field from the same film as Fig. 1. The vacuoles are larger and more easily seen, and nuclear displacement is very evident. × 400.

*Fig.* 3.—Another clump of cells from the sputum of Case 1. There is less vacuolation than is shown in the previous illustration, but considerably more nuclear pleomorphism. × 400.

*Fig.* 4.—A clump of cells found in the sputum of Case 2. The cells surround a number of vacuoles and the larger mass is hollow. The globular nature of this clump could be demondstrate by racking the objective of the microscope up and down, but cannot adequately be shown in a photograph.
This type of appearance, though commonly seen in carcinomatous pleural effusions or ascitic fluids, is very rare in sputum. × 400.



Philps.

It has been stated in the previous paper that vacuolation of the cytoplasm can occur in single non-malignant cells, but in my experience, vacuolation in the cells composing a fragment of tissue is seen only in carcinoma.

*Case 2.*

This case is not so well documented as the previous one. The patient was a woman of 64 who showed miliary shadows in the lung fields.

Clumps of cells, one of which is shown in Fig. 4 were seen in her sputum, which was reported as follows : " Mucoid sputum. Films show clumps of cells which constitute clear evidence of carcinoma. They are of adenocarcinomatous type and do not resemble cells usually seen in primary bronchial carcinomata." An enlarged cervical gland was later removed for histological examination and was reported as showing a mucus-secreting adenocarcinoma, probably of colonic origin. The patient died, and post mortem examination was refused.

It will be noted that the cells shown in the clump in Fig. 4 appear to surround a number of vacuoles, but gross vacuolation is not evident in the cells themselves. The larger mass is hollow, the wall being formed of a single layer of cells.

I make it a practice only to report adenocarcinoma from sputum examination when clumps of the types described are seen. It cannot be too strongly emphasised that single cells containing vacuoles as shown in Fig. 38 of the previous communication do not imply carcinoma.

I am indebted to A. Bligh, A.I.B.P. for the four photographs.

REFERENCE.

PHILPS, F. R. (1954) *Brit. J. Cancer*, **8**, 67.

# APPEARANCES WHICH MAY CAUSE CONFUSION IN THE DIAGNOSIS OF BRONCHIAL CARCINOMA FROM SPUTUM EXAMINATION.

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THE examination of sputum for carcinoma cells can only be considered a diagnostic method of clinical value if false positive diagnoses be avoided and cases wrongly thought suggestive of carcinoma reduced to a minimum. The method cannot be used to exclude carcinoma as it is evident that malignant cells may not be found if they are present only in small numbers, or in the case of an early growth the sputum may contain none. It must be accepted, therefore, that 20 to 25 per cent of bronchial carcinomata will be missed by this method and the problem is to be sure that a positive report is as reliable as possible. The descriptions and illustrations presented in this paper are of appearances considered sufficiently misleading to be a possible source of error.

I can find few illustrations in the literature of appearances that have mistakenly been thought due to carcinoma, but Wandall (1944) publishes photographs of atypical macrophages, giant cells, and clusters of epithelioid cells, and draws attention to the misleading appearances which these sometimes present. In a previous communication (Philps 1954*a*), photographs were published of a number of appearances that had led to confusion, namely cells showing considerable pleomorphism (Fig. 35), a cell enclosed within a macrophage (Fig. 36), cells showing large nuclei with coarse chromatin network (Fig. 37), single cells with vacuolated cytoplasm (Fig. 38), epithelial cells of unusual appearance from neutrophil infiltration of the cytoplasm (Fig. 39), a body that was thought to be the centre of an epithelial pearl (Fig. 40), and epithelial cells in unusual arrangement (Fig. 41 and 42). In addition, a drawing was published of a corpus amylaceum enclosed within a macrophage, suggesting on superficial examination, a squamous carcinoma cell with a large dense nucleus (Fig. 43).

The appearances which form the subject of the present communication have been collected over a period of three years, and though in no case was a positive diagnosis of carcinoma made from the sputum film, two were considered suggestive of it. One of these films is shown in Fig. 1, 2, 3, 15 and 16, and the other in Fig. 12 and 13. In only one case in the present series was the patient in fact suffering from bronchial carcinoma and there was ample evidence of this in the sputum. Fig. 6 and 7 show a group of cells from the sputum of this patient which, for reasons which will be given later are clearly not malignant. I feel therefore that the inclusion of this patient in the series is justified.

The appearances to be described may be divided into the following groups:

(*a*) Appearances associated with unusual epithelial breakdown and activity (Fig. 1, 2, and 3).

(*b*) Appearances due to bronchial epithelial cells in unusual arrangement (Fig. 4, 5, 6 and 7).
(*c*) Appearances due to epithelial cells whose precise origin cannot be determined (Fig. 8, 9, 10 and 11).
(*d*) Appearances associated with bronchial adenoma (Fig. 12 and 13).
(*e*) Appearances due to macrophages of unusual type (Fig. 14, 15 and 16).
(*f*) An appearance shown also in the drawing already mentioned (Philps, 1954*a*), due to the inclusion of a corpus amylaceum within another cell (Fig. 17).

(*a*) *Appearances associated with unusual epithelial breakdown and activity.*

In the earlier paper (Philps, 1954*a*) it was stated that mitosis is rarely seen in carcinoma cells in the sputum and is not considered a reliable guide to malignancy. The sputum film shown in Fig. 1, 2 and 3 contained a considerable amount of necrotic epithelial tissue, and in one small group of cells, two mitotic figures, one of which is shown in Fig. 1 and 2. The cells in mitosis appeared to be of squamous type, as did those breaking down (Fig. 3) and the film was reported as being suggestive of carcinoma. In fact, the patient was suffering from pulmonary tuberculosis and has shown no evidence of carcinoma in the period of 15 months since the examination was made. It was this film which demonstrated to me the unreliability of mitosis as an indication of carcinoma in scattered cells of epithelial type.

(*b*) *Appearances due to bronchial epithelial cells in unusual arrangement.*

Bronchial epithelial cells may give rise to confusion because they occasionally form clumps in the sputum. Two such clumps are seen in Fig. 4, 5, 6 and 7. In both cases the appearance suggested a clump of carcinoma cells but the ciliated border could in places be clearly seen, making the true nature of the cells evident.

Bronchial epithelial cells sometimes show vacuolation of the cytoplasm and may then give a disorderly appearance with may lead to confusion with carcinoma cells. Such an appearance is illustrated in Fig. 4 of the paper already mentioned (Philps, 1954*a*). The significance of vacuolation of the cytoplasm in the identification of carcinoma cells is discussed in that paper and in a subsequent one (Philps, 1954*b*).

(*c*) *Appearances due to epithelial cells whose precise origin cannot be determined.*

Clumps of small epithelial cells, probably derived from the basal layer of the bronchial epithelium are occasionally seen in the sputum and may give an appearance resembling cells from anaplastic carcinomata of oat cell type. Fig. 8, 9, 10 and 11 show two such groups of cells. That shown in Fig. 8 is particularly confusing, as the cells are held together in a clump by strands of mucus making the mass similar in shape and proportions to a typical clump of oat cells. In both cases the cells could be distinguished from oat cells by the amount of cytoplasm they possessed and by the absence of any moulding between the nuclei of neighbouring cells. I consider it unwise to diagnose oat cell carcinoma unless the following criteria be satisfied, namely:

(1) The individual cells are about the size of a lymphocyte.
(2) Cytoplasm is either not seen, or is at most a very thin rim round the nucleus.

## DESCRIPTION OF PLATES.

All films were stained with haemalum and eosin.

FIG. 1, 2 and 3.—Cells from a film in which there was a considerable amount of tissue destruction and some mitotic figures, from the sputum of a patient suffering from pulmonary tuberculosis.

FIG. 1.—An area showing necrotic debris, amongst which was a cell of epithelial type in mitosis. × 400.

FIG. 2.—A higher magnification showing the mitotic figure. × 1500.

FIG. 3.—Another field from the same film showing necrotic cells which appear to be of squamous type. × 400.

FIG. 4, 5, 6 and 7.—Confusing appearances associated with ciliated epithelial cells.

FIG. 4.—A circular clump of ciliated epithelial cells. Circular clumps of carcinoma cells may occur, giving an appearance not unlike this. In this case, the ciliated border of the cells made their benign nature clear. × 400.

FIG. 5.—The edge of the same clump under higher magnification to show the cilia. From the sputum of a patient suffering from pulmonary tuberculosis. × 1500.

FIG. 6.—Part of a group of cells of disorderly appearance rather suggestive of carcinoma, though the nuclei are more regular than is usual in malignant cells. A few of the cells, which appear clearly to be of the same type as the others in the group, show a ciliated border and it is therefore reasonable to assume that all the cells in the group are non-malignant. × 400.

FIG. 7.—Part of the group shown in the previous figure under higher magnification to show the cilia. This patient was in fact suffering from bronchial carcinoma and had ample evidence of this in his sputum. × 1500.

FIG. 8, 9, 10 and 11.—Confusing appearances due to groups of cells of uncertain origin.

FIG. 8.—A clump of cells apparently held together by mucus, giving a superficial resemblance to oat cells. On close inspection of this clump, it was clear that the individual cells did not form part a fragment of tissue as there were spaces between them, and they had more cytoplasm than do oat cells. From the sputum of a patient suffering from haemorrhagic bronchitis. × 400.

FIG. 9.—Part of the clump of cells in Fig. 8 under greater magnification to show the relatively abundant cytoplasm. × 1500.

FIG. 10.—A group of cells which from their character were probably derived from the basal layer of the bronchial epithelium. They resemble oat cells. × 400.

FIG. 11.—Part of the group shown in the previous figure under higher magnification. The cells bear a strong resemblance to oat cells but can be distinguished from them because the cytoplasm is relatively abundant and the nuclei of adjacent cells show no tendency to mould together. From the sputum of a patient with haemoptysis resulting from pulmonary embolism. × 1500.

FIG. 12 and 13.—An appearance seen in a bronchoscope trap specimen from a patient suffering from bronchial adenoma.

FIG. 12.—The fragment of tissue composed of small cells with very densely staining nuclei. × 400.

FIG. 13.—Part of the fragment under higher magnification. The pattern of the cells resembles that seen in oat cell carcinoma, adjacent nuclei showing a tendency to mould together. There is more cytoplasm separating the nuclei than is commonly seen in oat cells, and the chromatin pattern is nowhere visible whereas in oat cells it is usually clearly seen. This appearance was reported as being suggestive of carcinoma. The diagnosis of bronchial adenoma was made at bronchoscopy and confirmed later at post mortem. × 1500.

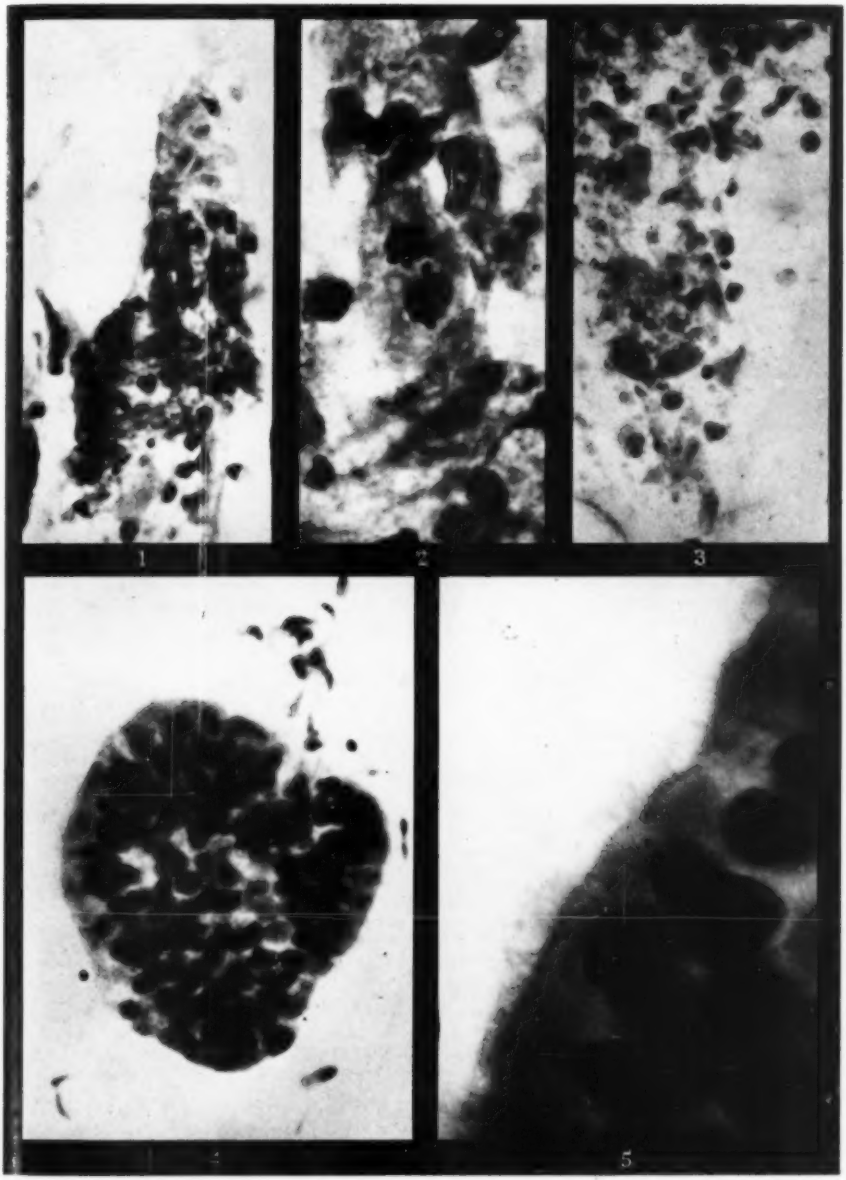
FIG. 14, 15 and 16.—Appearances due to macrophages of unusual type.

FIG. 14.—An elongated macrophage bearing a superficial resemblance to a "tadpole cell" of the type commonly seen in squamous cell carcinoma. The cell is clearly a macrophage as it is identical, apart from its shape, with macrophages lying nearby, and it contains minute dust particles. This patient was not suffering from any pulmonary disease. × 400.

FIG. 15.—A multinucleate giant cell from the sputum of the same patient as is shown in Fig. 1, 2 and 3. The cytoplasm is foamy and there were 12 or more nuclei in the dense central part of the cell. The patient suffered from tuberculosis. × 400.

FIG. 16.—A photograph taken at a higher power and at greater penetration to show the nuclear detail of the cell shown in Fig. 15. × 800.

FIG. 17.—A corpus amylaceum enclosed within a cell which appears to be of epithelial origin. The nucleus of the enclosing cell was at the periphery of the enclosed body but is not well shown in the photograph. The corpus amylaceum was semitransparent and was devoid of any nuclear characteristics. When stained with methylene blue, such a body stains very deeply and might be confused with a large deeply stained nucleus. More usually, corpora amylacea are circular. From a patient suffering from aspiration pneumonia. × 400.

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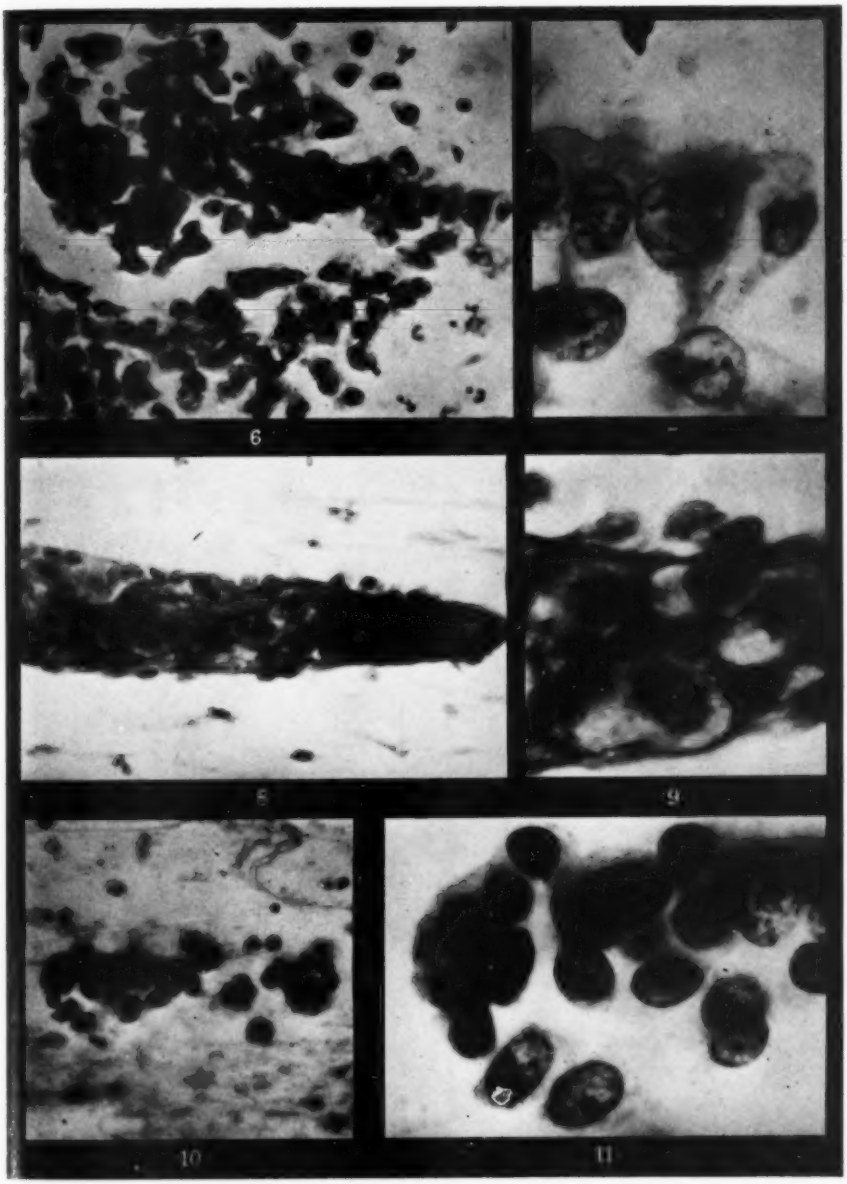

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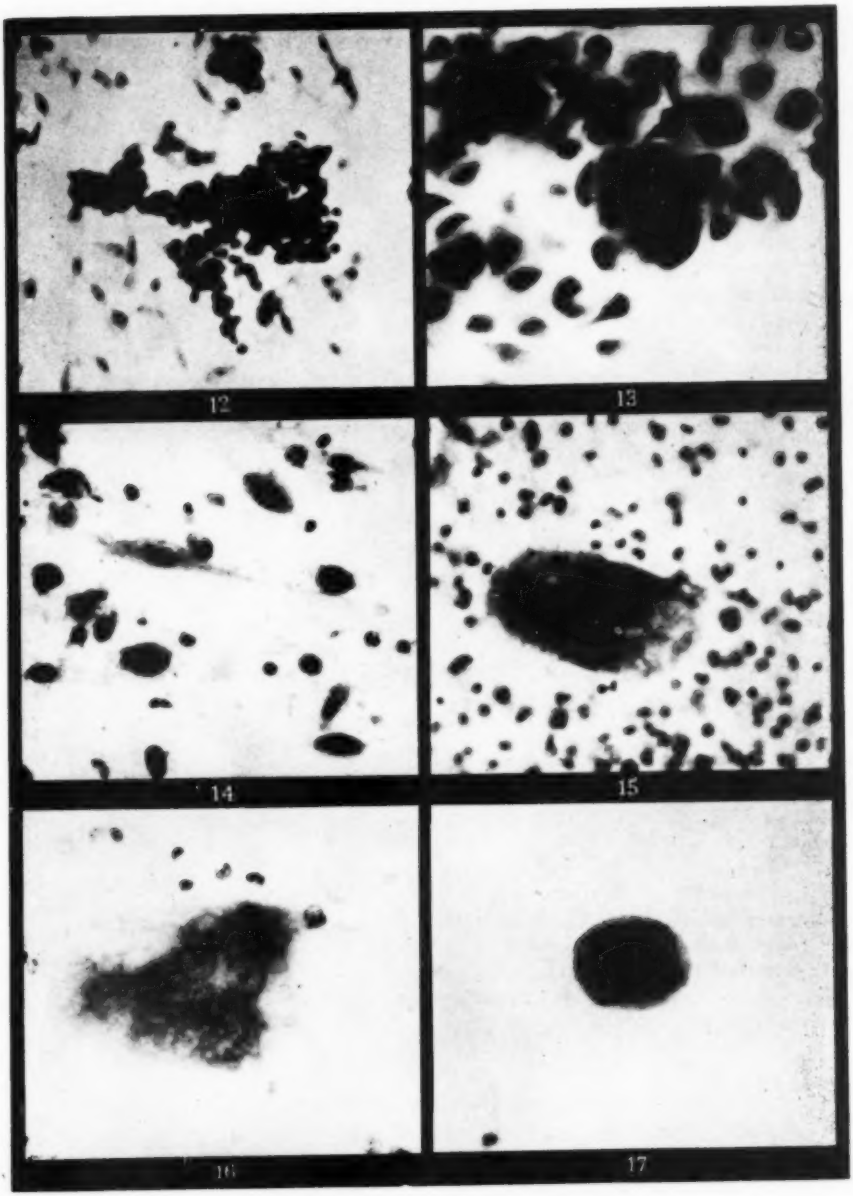


Philps.

(3) The nuclear chromatin of many of the cells forms a loose network reminiscent of a reticulocyte stained to show the reticulum, though dense nuclei may also be seen.
(4) The nuclei of some adjacent cells are seen to mould and indent each other.

(*d*) *Appearances associated with bronchial adenoma.*

Fig. 12 and 13 show a fragment of tissue from a bronchoscope trap specimen taken from a patient with a bronchial adenoma. The nuclei of the individual cells mould together rather as do those of oat cells, giving the whole clump an appearance resembling oat cell carcinoma. All the nuclei however, are dense and the cells possess more cytoplasm than is usually seen in oat cells. The specimen was reported as follows: "Films show clumps of cells, the arrangement of which is suggestive of oat cell carcinoma though the individual cells are not sufficiently characteristic to indicate malignancy." The diagnosis of bronchial adenoma was made at bronchoscopy and confirmed at post mortem nine months later following the patient's death from coronary thrombosis.

(*e*) *Appearances due to macrophages of unusual type.*

Macrophages, when they are seen to contain dust particles, can be easily identified, but when devoid of such particles, they may sometimes be confused with carcinoma cells. A macrophage which has engulfed another cell may resemble some types of cells seen in squamous cell carcinoma. It is therefore unwise to diagnose carcinoma upon this appearance unless there is other evidence that the cells are in fact malignant. This point has already been discussed (Philps, 1954*a*). It was also stated then that a macrophage may occasionally assume an elongated form and closely resemble a "tadpole cell" of the type often seen in squamous carcinoma (Philps, 1954*a*, Fig. 13 and 14). A macrophage of this type is seen in Fig. 14 of the present paper. Its nature is clear from the resemblance in nuclear and cytoplasmic characteristics that it bears to macrophages of more usual shape nearby, but the possibility of confusion must always be borne in mind when squamous cell carcinoma is diagnosed from cells of "tadpole" type.

Macrophages may on occasion be seen to compress and indent each other in clumps in rather the same way as do oat cells. This may be a source of confusion in a film stained with methylene blue in which macrophages sometimes stain densely, but in haemalum and eosin stained films, difficulty should not arise as the characteristics, particularly the amount of cytoplasm, of macrophages are quite different from those of oat cells. In addition, during fixation for the permanent film, macrophages tend to contract away from each other, losing the appearance of being compressed together, whereas oat cells do not, presumably because they form part of a fragment of tissue.

Fig. 15 and 16 show a multinucleate giant cell which appears, from the foamy nature of the cytoplasm, to be of the macrophage type. The nuclei are central, and number about 12. Multinucleate cells frequently occur in carcinoma but they are in no way diagnostic of it, the commonest multinucleate cell seen in sputum films being the macrophage. This example is the largest that I have seen. The patient was suffering from pulmonary tuberculosis but the cell does not appear

to be a Langhans giant cell. Osborn (1953) publishes a good photograph of a giant cell.

(*f*) *An appearance due to the inclusion of a corpus amylaceum within a cell.*

As has been stated, a drawing has already been published of a corpus amylaceum enclosed within a cell, giving a superficial impression of a cell with a large nucleus which might be thought to be malignant. It is only in the methylene blue preparation that such an impression may be created, as corpora amylacea do not stain densely with haemalum and eosin. However, since confusion might arise, Fig. 17 is included to show the appearance in a film stained by the latter method. The absence of nuclear characteristics in a corpus amylaceum should make its identity clear. Almost always, these bodies are circular. This one is exceptional in its irregular shape.

SUMMARY.

Descriptions and illustrations are presented of some appearances seen in sputum films which may erroneously be thought to be due to carcinoma.

I am indebted to A. Bligh, A.I.B.P., for the photographs.

REFERENCES.

OSBORN, G. R.—(1953) ' Applied Cytology.' London (Butterworth & Co. Ltd.), p. 139.
PHILPS, F. R.—(1954*a*) *Brit. J. Cancer.* **8**, 67.—(1954*b*) *Ibid.*, **8**, 240.
WANDALL, H. H.—(1944) *Acta. chir. scand.*, Suppl. 93. 1.

# THE DIAGNOSIS OF CARCINOMA OF THE PANCREAS.

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THE earliest recorded description of carcinoma of the pancreas is attributed to Mondière in 1836. Da Costa (1858) of Philadelphia reviewed 37 cases and gave a comprehensive description of the clinical presentation of this disease with emphasis on pain as a prominent sympton in 32 of these patients. He pointed out that " It (the pain) may be suddenly augmented by turning in bed from side to side. In not a few cases it is increased by the erect position and hence we find patients seeking relief by stooping and curving their body forward so as to relax the abdominal parietes."

Physicians of the French school sustained their interest in this condition, and Bard and Pic in 1888 observed the common association of a palpable gall bladder with jaundice, and the frequent incidence of cachexia. From the descriptions given by these and other observers the concept of painless jaundice as the classical form of presentation of this disease spread widely.

Mirallie (1893) found glycosuria in 3 cases, and Chauffard (1908) emphasized distinctive features of growths involving the body of the gland.

Speed (1920), Eusterman (1922), Eusterman and Wilbur (1933), Kiefer (1927) and other observers have subsequently reported on series of cases, and Berk (1941) has included these in his review of a total of 1449 cases. Levy and Lichtman (1940), and Duff (1939) have further clarified the picture of carcinoma of the body and tail of the pancreas, emphasizing early and severe pain, ascites, venous thrombosis, and widespread metastases to liver and peritoneum as important features.

Towards the end of the last century surgeons became interested in the pancreas, and Finney (1910) quotes a case of total pancreatectomy for carcinoma by Billroth in 1884 with recovery of the patient from the operation. Gordon-Taylor (1934) successfully removed a carcinoma of the body of the pancreas in 1927, his patient surviving when the case was reported 7 years later. In spite of such isolated successes, however, attempts at surgical removal gave little promise until, in 1935, Whipple and his colleagues demonstrated the technical feasibility of resection of the head of the gland, and opened a more hopeful era (Whipple, Parsons and Mullins, 1935; Whipple, 1938).

These and subsequent advances in surgery have greatly intensified the need for early diagnosis in a disease which has been estimated to comprise 1 to 2 per cent of all cancers, and which is by no means a clinical rarity. The insidious onset of the disease in many cases, its anatomical and radiological inaccessibility, and the tendency to early extension beyond the confines of the pancreas combine to constitute a stern challenge to the clinician.

* Holder of a Travelling Bursary awarded by the Board of Governors of Westminster Hospital.

The present series consists of 46 cases, confirmed at operation, in the wards of the Graduate Hospital of the University of Pennsylvania during the period July, 1940, to November, 1951. These cases are analysed from the clinical, radiological and laboratory standpoints on admission to hospital, in an attempt to present the combined diagnostic features of carcinoma of the pancreas.

*General considerations.*

*Anatomical site of the tumour.*—The head of the gland was involved in 31 cases (67·4 per cent), of which 6 cases extended to the body and 2 involved the whole gland. The head was spared in 14 cases (30·4 per cent), of which 4 cases involved the body alone, 4 cases the tail alone, and 6 cases both regions. The site in one case was not clearly identified.

*Age and sex distribution.*—The average age of the patients was 59·9 years with a range of 37 to 79 years. Other series have reported 56·4 years (Berk, 1941), 55·9 years (Dashiell and Palmer, 1948), and 58·2 years (Broadbent and Kerman, 1951). Extremes of 18 to 91 years have been recorded.

There were 20 females in this series, giving a ratio M : F, 1·3 : 1, a higher female incidence than usually found. Berk's (1941) figure of M : F, 2·4 : 1 is probably more representative.

*The symptomatology.*

The average duration of symptoms before admission to hospital was 4·16 months. Broadbent and Kerman (1951) found that 7·5 months elapsed on average in their series. These two figures are an index of the very real diagnostic difficulties encountered. It may be argued that such a delay is comparable to that encountered in carcinoma of the stomach, a disease far more accessible to the radiologist, but in this interval the balance swings towards inoperability.

Pain was far and away the most frequent initial symptom, occurring in 29 cases (63 per cent), and jaundice a poor challenger with an incidence of only 9 cases (19·5 per cent). General weakness occurred initially in 4 cases (8·7 per cent), anorexia in 3 cases (6·5 per cent), nausea, constipation and diarrhoea each in 2 cases (4·3 per cent). Venous thrombosis in the legs occurred initially in 2 cases (4·3 per cent), and the only case of islet-cell carcinoma in the series presented in coma.

When the total presenting symptoms up to the time of admission to hospital were analysed, a different picture emerged. Pain was still ahead with an incidence of 84·7 per cent and loss of weight (to an average of 23·9 lb.) close behind with a frequency of 80·3 per cent. Jaundice occurred in 67·5 per cent and was painless in only 13 per cent of cases. The other main complaints were anorexia (54·3 per cent), nausea or vomiting (19·5 per cent) and diarrhoea (8·7 per cent).

It is suggested, therefore, that pain is the dominant symptom in pancreatic carcinoma and of great importance as a guide to early diagnosis. Likewise weight loss of considerable degree in the absence of obvious cause should focus thought upon the pancreas. The symptom of jaundice is clearly one of great importance, but the concept of it (especially in its painless form) as the chief and classical signpost to a diagnosis of carcinoma of the pancreas can be grossly misleading.

An uncommon but important association with this disease is that of psychological disturbances, usually with depressive or apathetic features, and such

occurred in 3 cases in the present series. Yaskin (1931) and Latter and Wilbur (1937) have described this association, and it is of special importance to remember it in the presence of undiagnosed abdominal or back pain. Such a patient may appear markedly neurotic, partly, perhaps, because no one seems to understand his complaint, and this can lead to a tragic misdiagnosis in the presence of a painful disease.

*A consideration of pain in pancreatic carcinoma.*

An attempt at correlation between the site of the tumour and the site of pain produced is summarised in Table I.

TABLE I.—*Correlation between Site of Tumour in Pancreas and Site of Pain.*

| | Back (per cent). | Epigastric (per cent). | Right upper (per cent). | Left upper (per cent). | Lower (per cent). |
|---|---|---|---|---|---|
| Of 31 cases involving the head | 25·8 | 45·2 | 38·7 | 12·9 | 6·5 |
| Of 14 cases involving body, tail or both | 64·3 | 78·5 | 18·0 | 18·0 | 21·4 |
| Of 4 cases involving tail alone | 75·0 | 75·0 | 25·0 | 0 | 50·0 |

Of 7 cases presenting without pain, the growth involved the head in 6, extending to the body in 2. The seventh, an islet cell carcinoma of the tail, presented in coma, and no clear history was obtainable. Excluding this latter case, every tumour involving the whole gland, the body alone, the body and tail, and tail alone gave rise to pain.

The incidence of right upper quadrant pain was higher in tumours involving the head. Epigastric and back pain occurred more commonly in growths involv- the body and tail. Left upper quadrant pain was relatively uncommon in any group, and growths involving the tail alone showed a marked tendency to give pain referred to the lower abdomen.

The postural relief and aggravation of pain as described by Da Costa (1858), was a marked feature in 8 cases, and in 4 cases pain of a peptic ulcer type, relieved by food and alkalis, occurred.

*Abnormal physical signs.*

Clinical jaundice was noted in 28 cases (60·8 per cent) on admission, and of these 17 cases had demonstrable hepatic enlargement, and 6 a definitely palpable gall-bladder. Three cases giving a history of recent jaundice were not noted to have clinical icterus on admission.

Of 19 non-jaundiced patients, only 4 had clinical enlargement of the liver, and none a definitely palpable gall-bladder.

Liver enlargement ranged from 3 to 6 finger breadths below the costal margin.

In 19 cases a mass considered to be the pancreatic tumour was palpable, and abdominal tenderness was noted in 14. One case had active thrombo-phlebitis, and 1 presented with paraplegia due to extradural metastases.

In many cases the disease was well-advanced by the time that abnormal signs were clear cut. The discovery at operation of a dilated gall-bladder that had defied clinical palpation occurred on several occasions.

*Investigations of particular value.*

1. *Radiological study with barium meal.*—Of 26 cases examined, a definite diagnosis was made in 11 (42 per cent), and some abnormality detected in 12 others (46 per cent). Abnormalities of stomach contour were noted in 11 cases. Deformity of the antrum with indentation on either curvature seen in the antero-posterior view (Fig. 1), and indentation of the posterior stomach wall seen in the lateral view (Fig. 2) were helpful features.

Abnormalities of contour or mucosal pattern occurred in the duodenal cap in 10 cases, and in the duodenal loop in 9. An example of widening of the duodenal loop is shown in Fig. 1. This area is generally recognised as one yielding classical diagnostic features, but it is also important to recognise early and minor changes. Fig. 3*a* and *b* illustrate a case in which a small abnormal segment at the beginning of the duodenal loop gave way to gross changes of disorganisation in the interval of 20 months elapsing between examinations (*a*) and (*b*).

In 3 cases changes were found in the 3rd and 4th parts of the duodenum. Fig. 4 illustrates a case of carcinoma involving the tail and body of the gland with indentation of the upper border of the 3rd part of the duodenum. Another helpful finding in 2 cases of tumour in this site was forward displacement of the duodeno-jejunal flexure. It is of interest that Broadbent and Kerman (1951) increased the percentage of their cases diagnosable on radiological grounds from 18·4 per cent to 53·9 per cent on retrospective review of the films. It is felt that the trained radiologist can often make the major contribution towards diagnosis.

2. *Tests of carbohydrate metabolism.*—Three cases in this series were known diabetics of many years standing. Four cases had diabetes diagnosed within 8 months of admission to hospital, and in these cases the diabetes may have been a manifestation of the progressive pancreatic lesion.

Repeated glycosuria occurred in 14 (32·2 per cent) of the total 46 cases. The fasting blood sugar was raised above 110 mg. per cent in 20 of 40 cases (50 per cent). Of 8 cases examined by the glucose tolerance test, a diabetic type curve was obtained in 6 cases (75 per cent). These latter cases included 2 of the known cases of diabetes of recent onset, but none of long standing. Two cases with normal fasting blood sugar and no glycosuria gave a diabetic type response to the glucose tolerance test. Dashiell and Palmer (1948) obtained a diabetic type curve in 85·7 per cent of 21 cases examined.

It is suggested that the glucose tolerance test is of great importance in the diagnosis of carcinoma of the pancreas, and that minor or moderate degrees of impairment of carbohydrate metabolism may escape detection by any less demanding test.

3. *Serum enzyme determinations.*—Serum lipase determinations (by the Loerenhart method modified by Cherry and Crandell, 1932) in 37 cases gave a value

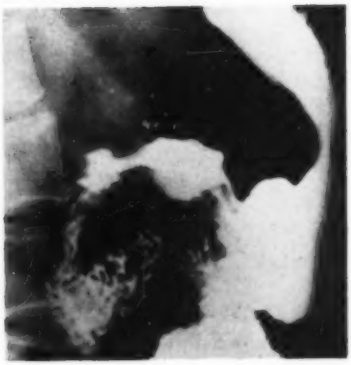
EXPLANATION OF PLATE.

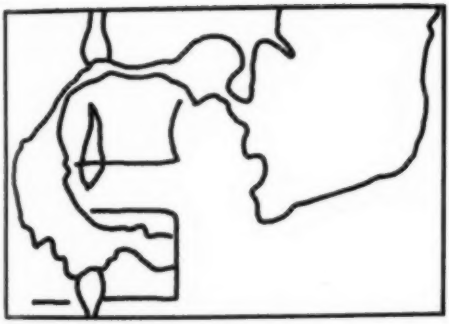
FIG. 1.—Indentation of the antrum of the stomach on the greater curvature, flattening of the greater recess of the duodenum, and widening of the duodenal loop in a case of carcinoma of the head of the pancreas.

FIG. 2.—This illustrates the value of the lateral view of the stomach in demonstrating indentation of the posterior wall by pancreatic growth.

FIG. 3.—This shows the significance of minor changes in the contour and mucosal pattern of the duodenum. 20 months elapsed between examination (*a*) and (*b*).

FIG. 4.—A case of carcinoma of the tail and body of the pancreas producing indentation of the upper border of the 3rd part of the duodenum.





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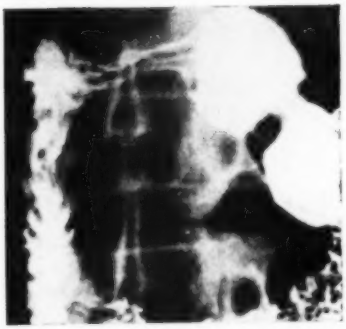

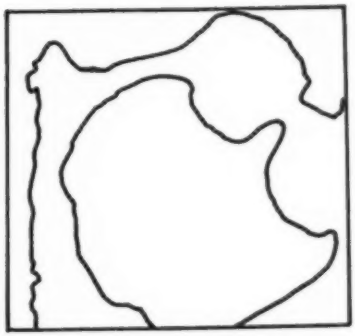

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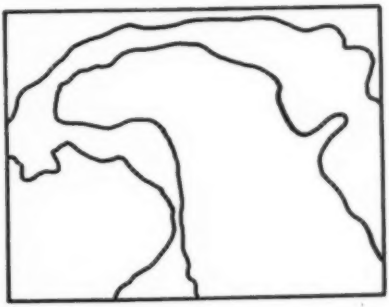
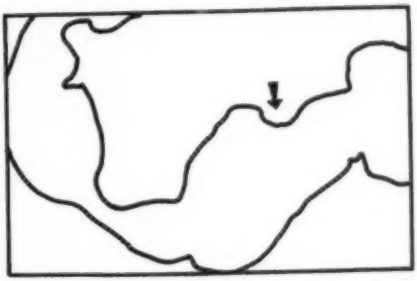


3b



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exceeding 1·0 c.c. of N/20 sodium hydroxide in 15 cases (40·5 per cent). Comfort and Osterberger (1940) reported an identical percentage positivity in their cases using a figure of 1·5 c.c. N/20 sodium hydroxide as the upper limit of normality. Johnson and Bockus (1940) reported a raised value in 5 of 8 cases.

Secretin stimulation of the pancreas was performed in 3 cases, accentuating an already raised level in 2 cases, and producing an abnormal value in 1. Of 27 jaundiced cases the serum lipase was raised in 12 and normal in 15, indicating that this test is independent of biliary obstruction.

The serum amylase was raised above 125 Somogyi units in 7 of 34 cases (20·6 per cent). On secretin stimulation a raised value was produced in 3 of 4 other cases. The amylase determination is therefore considered to be of less value than the lipase.

4. *The bromosulphalein retention test* was performed in 17 cases, all of which showed abnormal dye retention.

Of these cases, 9 had hyperbilirubinaemia, of which 3 were subsequently shown to have liver metastases. Five cases without hyperbilirubinaemia all had liver metastases at operation.

It is suggested that this test may be of value in indicating multiple liver metastates in the absence of biliary obstruction.

*Other investigations reviewed.*

The serum bilirubin was raised above 1 mg. per cent in 27 of 33 cases investigated, of which 24 had clinical jaundice.

The serum alkaline phosphatase was examined in 26 cases and was raised above 5 Bodansky units in 13, of which all had associated hyperbilirubinaemia. Of 13 cases shown to have liver metastases, the alkaline phosphatase was raised in 7 and normal in 6.

The serum cholesterol was raised above 220 mg. per cent in 20 of 26 cases, and hyperbilirubinaemia accompanied 18 of these 20 cases. A normal chloesterol level occurred in 5 cases with associated hyperbilirubinaemia. The cholesterolesters exceeded 50 per cent of the total cholesterol in 20 of 21 cases.

It appears, therefore, that these latter three investigations reflect only the presence of biliary obstruction, and beyond this are not helpful in the present diagnostic problem.

The plasma proteins were normal in 23 of 25 cases, there being some degree of hypoalbuminaemia in the remaining 2 cases.

The cephalin, thymol and colloidal gold flocculation and turbidity tests were normal in all of 22 cases examined. In 3 cases the cephalin flocculations test became positive after cholecysto-enterostomy. The value of these tests in the present connection is that they facilitate exclusion of hepatitis in cases presenting with jaundice, as pointed out by Maclagan (1947)

Of 46 cases examined, 31 (67·4 per cent) had haemoglobin levels below 13·0 g. per cent, with an average of 12·13 (76 per cent Hb.) for the whole series. The red blood cell counts ranged from 3·19 to 5·6 millions, with an average of 4·29 millions. No profound anaemia occurred.

The mild degree of anaemia encountered suggests that this feature may be valuable in the differential diagnosis from other forms of intra-abdominal malignancy.

## DISCUSSION.

In view of the inherent difficulty of diagnosis of carcinoma of the pancreas at a stage where surgical resection is possible, it is necessary that all means of investigation should be combined at the earliest opportunity.

Firstly the clinician must be alert to the modes of presentation of the disease and its substantial, and possibly increasing, incidence in the community. Once clinical suspicion has been aroused, a combined plan of investigation, including careful radiological study, a glucose tolerance test, serum enzyme determinations, and possibly a bromsulphalein retention test, offers considerable chance of providing evidence in support of such suspicion. Bourne (1936) has also advocated a stool fat analysis, and this might well be included.

The cytological examination of duodenal contents as reported by Lemon (1951, 1952) and Rubin, Palmer and Kirsner (1952) gives promise of further valuable help in diagnosis.

Should all investigations prove unrewarding and a strong clinical suspicion still remain, it would appear justifiable to proceed to surgical exploration, as has been advocated by Bourne (1936).

## SUMMARY.

The historical background of carcinoma of the pancreas is briefly reviewed, A series of 46 cases proven at operation is considered from the clinical, radiological and biochemical aspects in an attempt to clarify the helpful diagnostic features. The frequent incidence and importance of pain and weight loss as symptoms is emphasized. Jaundice is found to occur less frequently than either of the symptoms mentioned above, and to be painless in relatively few cases. A relationship between the anatomical position of the tumour and the site of the pain complained of by the patient is suggested.

A scheme of investigation is proposed which includes careful radiological studies, a glucose tolerance test, serum lipase and amylase determinations and a bromsulphalein retention test. Surgical exploration is advocated where strong clinical grounds alone exist.

I wish to express my warm thanks to Dr. H. L. Bockus, Professor of Gastroenterology in the Graduate Hospital of the University of Pennsylvania, for all his help and encouragement in this investigation. I am also grateful to Dr. A. Finkelstein for the X-ray studies, and to the members of the Medical and Surgical staff of the Graduate Hospital for permission to include cases under their care.

I wish also to thank Sir Stanford Cade for his kind help and criticism.

## REFERENCES.

BARD, L. AND PIC, A.—(1888) *Rev. Médecine*, **8**, 257.
BERK, J. E.—(1941) *Arch. intern. Med.*, **68**, 525.
BOURNE, G.—(1936) *Lancet*, ii, 1826.
BROADBENT, T. R. AND KERMAN, H. D.—(1951) *Gastroenterology*, **17**, 163.
CHAUFFARD, M. A.—(1908) *Bull. Acad. Méd., Paris*, **60**, 242.
CHERRY, I. S. AND CRANDELL, L. A.—(1932) *Amer. J. Physiol.*, **100**, 266.
COMFORT, M. W. AND OSTERBERG, A. E.—(1940) *Proc. Mayo Clin.*, **15**, 427.

DA COSTA, J. M.—(1858) *N. Amer. Medico-chir. Rev.*, **2**, 883.
DASHIELL, G. F. AND PALMER, W. L.—(1948) *Arch. intern. Med.*, **81**, 173.
DUFF, G. L.—(1939) *Johns Hopk. Hosp. Bull.*, **55**, 69.
EUSTERMAN, G. B.—(1922) *Trans. Amer. gastro-ent. Ass.*, **25**, 26.
*Idem* AND WILBUR, D. L.—(1933) *Sth. med. J., Nashville*, **26**, 875.
FINNEY, J. M. T.—(1910) *Ann. Surg.*, **51**, 818.
GORDON-TAYLOR, G.—(1934) *Ibid.*, **100**, 206.
JOHNSON, T. A. AND BOCKUS, H. L.—(1940) *Arch. intern. Med.*, **66**, 62.
KIEFER, E. D.—(1927) *Ibid.*, **40**, 1.
LATTER, K. A. AND WILBUR, D.—(1937) *Proc. Mayo Clin.*, **12**, 457.
LEMON, H. M.—(1951) *N.Y. St. J. Med.*, ii, 2155.—(1952) *Ann. intern. Med.*, **37**, 525.
LEVY, H. AND LICHTMAN, S. S.—(1940) *Ibid.*, **65**, 607.
MACLAGAN, N. F.—(1947) *Brit. med. J.*, ii, 197.
MIRALLIE, C.—(1893) *Gaz. Hop. Paris*, **66**, 889.
MONDIÈRE, J. T.—(1836) Quoted by Parmentier, E., and Chabrol, E. (1923), *Nouveau Traité de Médecine*, XV, 197.
RUBIN, C., PALMER, W. L. AND KIRSNER, J. B.—(1952) *Gastroenterology*, **21**, 1.
SPEED, K.—(1920) *Amer. J. med. Sci.*, **160**, 1.
WHIPPLE, A. O.—(1938) *Amer. J. Surg.*, **40**, 260.
*Idem*, PARSONS, W. B. AND MULLINS, C. R.—(1935) *Ann. Surg.*, **102**, 763.
YASKIN, J. C.—(1931) *J. Amer. med. Ass.*, **96**, 1664.

# TRANSPLANTATION OF HUMAN TUMOURS INTO MOUSE-BRAIN.

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THE maintenance of human tumours under artificial conditions is a problem which has tested experimental workers for many years. Heterologous transplantation into different animals has been considered by most observers as unlikely to succeed for a variety of reasons, among which may be quoted the essential specificity of the protein make-up of cells in any particular animal species and the immune reaction leading to their destruction which is likely to be set up on their introduction into a heterologous strain. Failure of growth in new surroundings is characterised by lymphocytic infiltrations, proliferations of fibroblasts, collagen formation and failure of vascularisation. Loeb (1930) felt that death of a transplant within a few days of inoculation was due to immune responses by connective tissue elements at the site rather than secondary development of humoral antibodies. Sites for inoculation were sought where blood supply and connective tissue capable of immediate granulomatous response against a foreign body were minimal and where it might be hoped that foreign cells would become established sufficiently in their new environment to withstand vascularisation and subsequently to gain nourishment from it in order to proliferate.

As early as 1873 Van Dooremaal suggested that the anterior chamber of the eye might provide a suitable site for inoculation and in 1884 Zahn attempted to transplant a human chondroma in this way. It became infiltrated by lymphocytes and was absorbed.

Subsequent experiments seemed to confirm the early fears of failure and most workers formed the view that whilst homologous transplantation—that is transference into a like species—was sometimes possible, particularly when a rapidly growing tumour was employed, heterologous transplantation would prove uniformly unsuccessful.

In 1937 Smirnova reported successful transference of a human mammary carcinoma, and Greene with co-workers, in a series of papers from 1938 up to the present time, has described successful results of heterologous transplantation of human tumours, usually into the anterior chamber of the eyes of guinea pigs, although other animals such as rabbits have been used.

The results obtained by Greene have been more successful than those reported by other workers and he has developed the thesis that the ability of tumours to survive in heterologous species may be a measure of the ability of the malignant cell to metastasise. He recognises two groups of tumours from the point of view of transplantability, the difference between which is not based on the morphology of the neoplasm, but on the results of their behaviour in the patient. Thus he describes two groups of tumours, one of which contains hetero-transplantable material obtained from patients whose disease follows a rapidly fatal course,

and the other consists of material from patients who live for considerable periods of time. He goes further to suggest that when a tumour becomes capable of metastasis it also becomes possible to transplant it successfully into heterologous species. (Greene, 1952).

This view is supported by Lemon and Parsons (1952) who base their opinion on a small series of cases; but Towbin (1951*a*) reporting on the results of one hundred different human tumours, and thirty-eight different animal tumour transplants, was unable to confirm these findings to his satisfaction and concluded that relatively few human tumours, even though clinically malignant, can be transplanted successfully to the anterior chamber of the guinea pig's eye. He felt that this factor precludes the use of the technique as a practical measure for differentiating benign from malignant tumours. Morris, McDonald and Mann (1950) agree with these conclusions. After attempting to transplant forty different human malignant tumours they decided that successful heterotransplants into the anterior chamber of the eye can "seldom be accomplished." It was their view, however, that more successful results could be obtained if the tumour was introduced into the lens.

Although the anterior chamber of the eye has been the most popular site for attempted heterotransplantation, many other anatomical areas have been used, most of which have proved unsuccessful, with the exception of the brain in mice and the cheek pouch in hamsters. Greene (1951) has reported successes with mouse-brain inoculation and Lemon and Parsons (1952) have reported early successes in the hamster cheek pouch.

In addition to the search for a suitable site for tumour transplantation various attempts have been made to assist growth in the new surroundings. Most of these attempts have been designed to counteract or inhibit the immune reaction on the part of the host. Pre-operative irradiation of the host animal in order to reduce circulating lymphocytes has been tried by Toolan (1951) with apparent improvement in the transplanted tumour growth. Cortisone and A.C.T.H. have been administered but so far few reports of results are available. Several workers, among whom may be mentioned Toolan (1951), Lemon and Parsons (1952), and Dobyns and Lennon (1952), have tried serial transplantation of human tumours, and the last named authors describe a single case of a thyroid carcinoma which they have maintained by serial transplantation over a period of $3\frac{1}{2}$ years. In addition they record the interesting fact of an increasing degree of histological differentiation in the tumour during this period.

It has been suggested that the criteria for assessing continued viability of a tumour in its new surroundings are not sufficiently accurate and that the demonstration of cells which are alive as judged by histological means, or an increase in total size of a tumour, does not necessarily indicate a "take." Towbin (1915*b*) has pointed out in relation to transplantation to guinea pig anterior chamber that gross appearances may be deceptive and must be substantiated by histological studies. He has suggested the analysis of transplantation results in three stages.

1. A stage of suspension which may be compared to the conditions of tissue culture where the aqueous humour acts as a temporary nutritional medium.

2. A stage of nidation where the transplanted tumour elements become integrated with the vascular system of the new host. At this stage death may occur, or if the transplantation is successful, growth will ensue. In many cases Towbin (1951*b*) considers that a period of growth arrest may develop where no change

takes place in the tumour transplant and this stage may be maintained for very long periods.

3. A stage of growth when, if transplantation has been completely successful, the tumour proliferates.

The use of mammalian or avian brain as a focus for the transplantation of heteroplastic tissue seems to have been recorded first by Shirai in 1921 who reported briefly that tumours from rats had been grown successfully in the brains of chickens, pigeons and other animals. Murphy in 1926 presented a careful account of the transplantation of mouse sarcoma and carcinoma into rat brain. He recorded satisfactory and rapid growth up to periods of 3 to 4 weeks.

It was his opinion that failure of growth of a heteroplastic graft was not due to any lack of suitable nutrient material or to the formation of specific antibodies. The evidence available was, he felt, that the cellular reaction was of the first importance in determining the fate of a heteroplastic graft. The accumulation of small round cells around foreign material introduced into the higher animals has been well recorded, and it was Murphy's (1926) view that where this reaction is absent as in the embryo or in the brain, the heteroplastic graft meets with no resistance and its rate of growth is at least as great as it would be in the native host. He felt that all his evidence pointed to the importance of the lymphoid type of cell in the resistance to foreign tissue, and he performed experiments of reducing total lymphocytes by X-irradiation and by adding lymphocytic material, usually in the form of fresh spleen, along with the implanted tissue, the results of which he felt confirmed his theory.

In view of the relatively small number of reports in the literature of the results of heterotransplantation of human tumours it was decided to commence a series of experiments. A start has been made using the mouse-brain for inoculation as this site appeared to have been the least studied, but later the anterior chamber in guinea pigs has been used and more recently still, experiments have been commenced in which the cheek-pouch of the hamster has been utilised. This presentation is a report of the results obtained following transplantation of sixty different human tumours into mouse-brain and the account will be confined largely to changes taking place during the first 4 to 5 weeks following inoculation.

## Technique.

Malignant human tumours are obtained from Westminster Hospital. The portion for inoculation is selected under aseptic conditions in or near the operating theatre immediately after removal. An attempt is made to take a piece of tumour with as little stroma as possible and also to avoid obvious areas of necrosis or haemorrhage. The specimen is placed in a sterile container and transported at once to the laboratories of the Imperial Cancer Research Fund at the Royal College of Surgeons of England. The time interval at best is in the region of 20 minutes, but delays up to an hour are sometimes unavoidable. Speed of transference is of vital importance in this operation and for this reason a series of experiments are to be performed where inoculation is done on the spot and the animals subsequently transferred to the laboratory. So far no attempt has been made to maintain the tumour at any particular temperature during the interval between its removal and its inoculation.

The animals used are mice of up to 10 weeks of age. In the light of the experience of previous workers, young animals are preferred, but in view of the irregular

availability of human tumour material it has been found impossible to insist on any very particular age. In the early stages of the experiment pure bred C3H mice were used, but later mixed strains have been accepted with no apparent effect on results.

Basal anaesthesia is employed (0·1 c.c. of a 0·016 per cent solution of nembutal per 10 g. weight of mouse) and is administered intraperitoneally. This gives quite rapid deep anaesthesia which lasts for 2 hours. It has the distinct advantage that a batch of mice can be prepared when the human operation is about to commence so that the experimental animals are ready for inoculation as soon as the tumour is available, thus cutting down the transference time interval.

The method of inoculation is to shave the skin of the mouse's head over the vertex and to make a small incision down to the bone. In the earlier experiments a small triangular bone flap was then raised which could be replaced at the end of the operation. This has been found unnecessarily complicated and time consuming, so that now a small hole is made with the end of a corneal scalpel. The skull of the mouse is thin yet rigid so that little difficulty is experienced in applying the correct pressure to produce a hole without damaging the underlying brain. Bleeding is usually minimal and can be stopped quickly by gentle pressure with a gauze swab.

A small fragment of tumour is then introduced into the brain either on the end of a blunted hypodermic needle, or, after rough mincing with scissors, it can be drawn into the needle and either pushed in with the needle stylette or forced in by pressure from an ordinary syringe. A No. 18 gauge hypodermic needle preferably provided with a guard at 3 mm. from its blunted point, has been found the most useful instrument for this procedure. Two points of importance emerge in connection with the technique of tumour introduction. Firstly, only a very tiny fragment of tumour material should be used. Larger pieces tend to undergo rapid necrosis. It is clearly impossible to define an exact size but the inoculum either minced or unminced should represent a piece of tumour certainly not more than 1 mm. in diameter. The second point of importance is to make sure that when the needle is removed from inside the skull, the tumour inoculum does not also come out. No great difficulty is experienced in achieving this, but a small practical point of importance is to make the line of needle entry oblique to the skull hole.

When inoculation is complete the skin is drawn together over the wound and sealed with collodion. The mouse is then replaced in its cage and within 12–18 hours returns to apparent complete normality. Mortality among the mice as a result of the operation is related directly to operation technique. In the earlier part of the experiment 20 mice were inoculated in each batch and 2 or 3 fatalities within the first 3 days were common. Now 10 mice are inoculated in each batch and a fatality is a matter of extreme rarity.

A portion of the tumour inoculated is fixed and examined histologically. When experiments were commenced an attempt was made to select a suitably cellular area of tumour by performing immediate frozen sections. No apparent advantage resulted from this, and it had the disadvantage of causing delay in inoculation so that it has now been abandoned.

The whole procedure of inoculation is performed using aseptic precautions. The operation takes place in a sterile chamber and sterile rubber gloves are worn by the operator. Until fully recovered from the anaesthetic mice are segregated,

but after 24 hours, inoculated batches are kept together under normal animal house conditions.

## RESULTS.

Throughout the experiment close observation of the inoculated mice has been maintained in order to observe any signs of cerebral compression resulting from tumour growth in the skull. No such signs appeared and it was early decided to kill the mice after specific intervals in order to examine the brains. The times selected for observation were at first 10, 15 and 21 days, but later when it was realised that in the absence of complete failure something would be seen in most cases at 10 days, the times were changed to 15, 21 and 28 days. More recently still, mice are killed at 15, 28 and 35 days. This report will be confined to observations made at these times, but from each batch some mice are sacrificed for serial transplantation experiments whilst others are kept indefinitely.

In the early stages of the experiment when technique had not been fully developed each tumour specimen was inoculated into 20 mice, but in order to economise in storage space 10 mice have been used since the time when operative mortality became negligible. Two mice are killed on each selected day and the remainder of the batch used for other purposes or kept indefinitely. The commonest cause of an operative death is haemorrhage into the brain substance. When the animal is killed the head is removed and a post-mortem performed on the remainder of the body. No example of metastasis from inoculated tumours has been discovered.

The skin is carefully stripped from the skull and although even by 10 days considerable or complete healing at the site of inoculation is found, it is usually quite easy to see where the tumour has been put into the skull up to several weeks afterwards. Next the bone is removed and the brain carefully extracted intact. Again it is usual to be able to see macroscopically the point of inoculation on the surface of the cerebrum. The cerebellum and pons are removed and in some cases where the tumour is obvious the cerebellum is trimmed towards the site. The remaining specimen is then fixed routinely for 24 hours in Formol-acetic (Suza's fixative as modified by Heidenhein). This fixative is found suitable as it preserves good cytological detail and at the same time tends to make the brain firm and so facilitates subsequent section cutting. Serial sections are then cut from each specimen although it is usual for the section cutter to be able to recognise the point in the block where he begins to cut through the tumour area. Every fifth section has been retained and of these, alternate ones are stained routinely by haematoxylin and eosin, leaving the intervening sections for further investigation if required.

The tumours which have been used up to date are listed in Table 1 and are seen to be made up of sixty different human cases which fall into the following groups:

| | Cases. |
|---|---|
| Carcinoma of breast | 27 |
| Squamous cell carcinomas | 8 |
| Melanomas | 6 |
| Ovarian carcinomas | 6 |
| Carcinomas of gastro-intestinal tract | 8 |
| Sarcomas | 3 |
| Carcinoma of lung | 1 |
| Carcinoma of kidney | 1 |

## TABLE I.

| No. | Age. | Sex. | Tumour type. | Metastases present or absent (+ or −). | Tissue used for inoculation. | No. of days up to which definite cells seen in mouse-brain. |
|---|---|---|---|---|---|---|
| 1 | 55 | F | Carcinoma breast | − | Primary tumour | Failure |
| 2 | 43 | F | " " | + but pre-op irr. | " " | " |
| 3 | 66 | F | " " | − | " " | " |
| 4 | 67 | F | " " | + Glands in axilla | " " | " |
| 5 | 62 | F | " " | + " " " | " " | 364 days |
| 6 | 45 | F | " " | + " " " | " " | 28 " |
| 7 | 48 | F | " " | − | " " | 15 " |
| 8 | 43 | F | " " | − | " " | Failure |
| 9 | 69 | F | " " | + Glands in axilla | " " | 21 days |
| 10 | 45 | F | " " | + " " " | " " | 15 " |
| 11 | 59 | F | " " | − | " " | Failure |
| 12 | 65 | F | " " | + " " " | " " | 15 days |
| 13 | 62 | F | " " | − | " " | Failure |
| 14 | 54 | F | " " | − | " " | 21 days |
| 15 | 62 | F | " " | − | " " | Failure |
| 16 | 47 | F | " " | + Glands in axilla | " " | 21 days |
| 17 | 55 | F | " " | + " " " | " " | 21 " |
| 18 | 57 | F | " " | + " " " | " " | 28 " |
| 19 | 49 | F | " " | + " " " | " " | 28 " |
| 20 | 72 | F | " " | + " " " | " " | Failure |
| 21 | 47 | F | " " | − | " " | 21 days |
| 22 | 43 | F | " " | + " " " | " " | 35 " |
| 23 | 52 | F | " " | + " " " | " " | 28 " |
| 24 | 45 | F | " " | + " " " | " " | 28 " |
| 25 | 45 | F | " " | + " " " | Metastasis | Failure |
| 26 | 57 | F | " " | − | Primary tumour | 28 days |
| 27 | 61 | F | " " | + " " " | Metastasis | Failure |
| 28 | 49 | M | Squamous cell carcinoma | Rec. ca. penis | Primary tumour | 28 days |
| 29 | 56 | M | Ditto | + Glands from tongue | Metastasis | 21 " |
| 30 | 48 | M | " | + Glands from larynx | " | 21 " |
| 31 | 66 | M | " | + Glands from tongue | " | 21 " |
| 32 | 51 | F | " | + Glands cervix uteri | " | 21 " |
| 33 | 42 | M | " | − Skin carcinoma | Primary tumour | 15 " |
| 34 | 64 | M | " | + Glands from tongue | Metastasis | 21 " |
| 35 | 52 | M | " | + " " " | " | 21 " |
| 36 | 31 | F | Melanoma | − | Primary tumour | Failure |
| 37 | 61 | M | " | − | " " | " |
| 38 | 52 | F | " | + Glands from groin | Metastasis | 21 days |
| 39 | 63 | F | " | + " " " | " | 28 " |
| 40 | 57 | F | " | + " in " | " | 28 " |
| 41 | 58 | M | " | + " " " | " | 28 " |
| 42 | 31 | F | Carcinoma ovary | + Omental spread | Primary tumour | Failure |
| 43 | 37 | F | " " | + Generalised | " " | 15 days |
| 44 | 61 | F | " " | + Krukenberg | Metastasis | 15 " |
| 45 | 50 | F | " " | + Generalised | " | 28 " |
| 46 | 49 | F | " " | + Omental spread | " | 15 " |
| 47 | 40 | F | " " | + Liver | " | 21 " |
| 48 | 64 | M | " stomach | + Adjacent glands | " | 21 " |
| 49 | 73 | F | " " | + Glands | Primary tumour | Failure |
| 50 | 73 | F | Carcinoma rectum | + " | " " | " (inf.) |
| 51 | 63 | F | " " | + " | " " | " " |
| 52 | 61 | M | " " | + " | Metastasis | 35 days |
| 53 | 62 | M | Carcinoma colon | + Adjacent glands | " | 21 " |
| 54 | 62 | M | " " | + " " | Primary tumour | 21 " |
| 55 | 49 | F | " " | + " " | " " | 28 " |
| 56 | 22 | M | Osteogenic sarcoma | − | " " | Failure |
| 57 | 79 | M | Fibrosarcoma | − | " " | 28 days |
| 58 | 23 | M | Osteogenic sarcoma | − | " " | 15 " |
| 59 | 54 | M | Carcinoma lung | − | " " | Failure (inf.) |
| 60 | 56 | M | Carcinoma kidney | − | " " | 35 days |

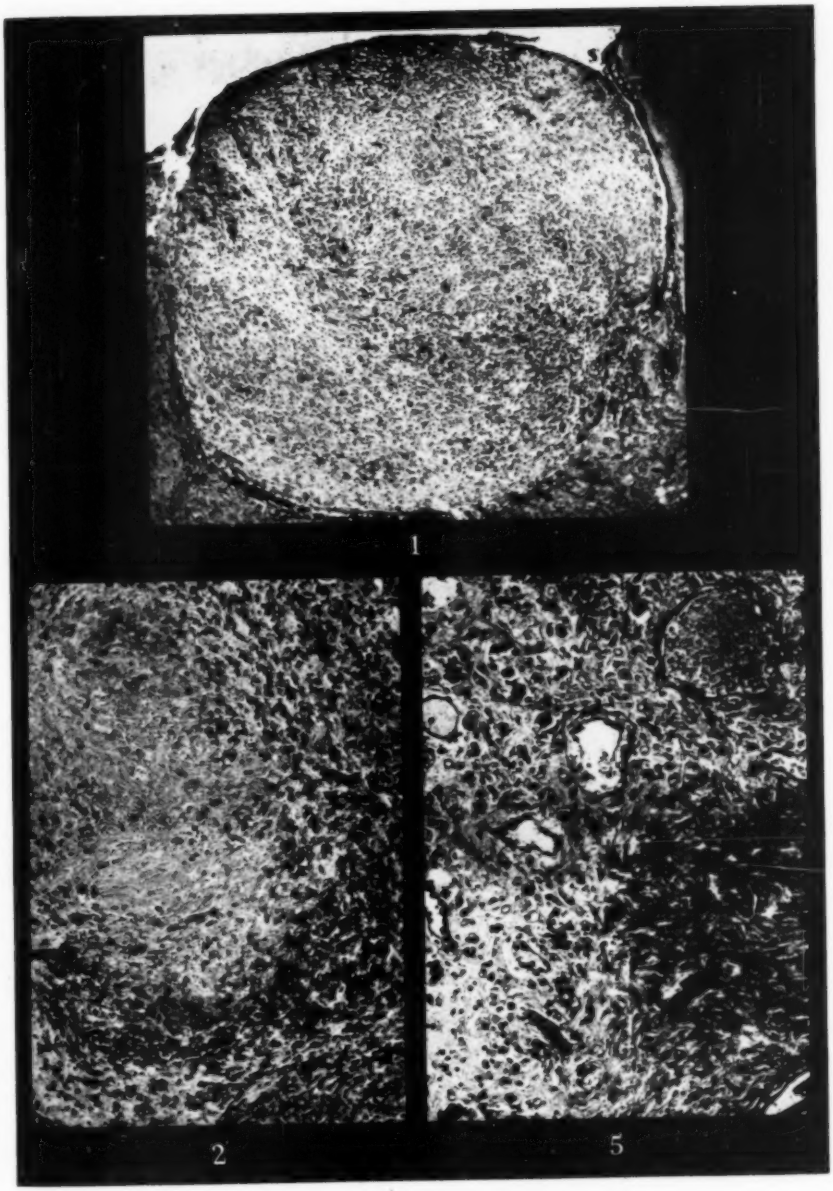
They are arranged in the table to show the age, sex and diagnosis of the patient from whom the tumour was taken, in addition to the result achieved on transplantation expressed in terms of number of days after inoculation at which tumour cells could be recognised by histological means.

In those cases where tumour cells are found they reproduce closely the histological appearances they presented when growing in human tissues and are readily recognisable (Fig. 6–15). The site of inoculation in the brain is clear cut and shows a sharp line of demarcation between inoculum and normal brain. The resulting growths following implantation tend to produce a discrete rounded nodule (Fig. 1) pushing aside the surrounding brain rather than infiltrating it. At 10 and 15 day periods necrosis of some of the tumour cells is seen. Sometimes maximum cell death is central in a nodule (Fig. 2) and more rarely it is the peripheral cells of a tumour clump which are mainly affected (Fig. 3). This death of cells is frequently associated with an infiltration of polymorphonuclear leucocytes (Fig. 3).

In brains examined at 21 days where some tumour cells have survived, inflammatory cellular infiltration is minimal both in the tumour nodule itself and also about its edges. Small vessels in the vicinity however, tend to be dilated and show perivascular "cuffing" by small round cells of lymphocyte type (Fig. 4). Frequently small vessels are seen plugged with lymphocytes. At 28 and 35 day periods in most of the cases examined in this series histologically identifiable

EXPLANATION OF PLATES.

FIG. 1.—Discrete rounded nodule of tumour well demarcated from surrounding brain. Transplant from metastasis from carcinoma ovary after 15 days. H. & E. × 55.
FIG. 2.—Central necrosis in group of cells from a fibrosarcoma after 15 days. H. & E. × 110.
FIG. 3.—Peripheral death of cells with well marked polymorphonuclear leucocyte proliferation from a squamous cell carcinoma 10 days after transplantation. H. & E. × 100.
FIG. 4.—Lymphocytic infiltration around small vessels at edge of inoculation area. H. & E. × 250.
FIG. 5.—Zone of inoculation where malignant cells have disappeared. Vascularisation and early fibrosis have taken place. H. & E. × 120.
FIG. 6*a*.—Carcinoma of breast, Stage II before inoculation. H. & E. × 260.
FIG. 6*b*.—Inoculated tumour in Fig. 6*a* after 21 days. H. & E. × 260.
FIG. 7*a*.—Carcinoma of breast, Stage II before inoculation. H. & E. × 250.
FIG. 7*b*.—Inoculated tumour in Fig. 7*a* after 15 days. H. & E. × 350.
FIG. 8*a*.—Carcinoma of breast, Stage II before inoculation. H. & E. × 240.
FIG. 8*b*.—Inoculated tumour in Fig. 8*a* after 21 days. H. & E. × 280.
FIG. 9*a*.—Metastasis in gland from squamous cell carcinoma before inoculation. H. & E. × 260.
FIG. 9*b*.—Inoculated tumour in Fig. 9*a* after 21 days. H. & E. × 80.
FIG. 9*c*.—Inoculated tumour in Fig. 9*a* after 21 days showing mitotic figure. H. & E. × 650.
FIG. 10*a*.—Metastasis in gland from melanoma before inoculation. H. & E. × 280.
FIG. 10*b*.—Inoculated tumour in Fig. 10*a* after 29 days. H. & E. × 280.
FIG. 11*a*.—Metastasis in gland from melanoma before inoculation. H. & E. × 280.
FIG. 11*b*.—Inoculated tumour in Fig. 11*a* after 21 days. H. & E. × 280.
FIG. 11*c*.—Inoculated tumour in Fig. 11*a* after 15 days invading ventricle. H. & E. × 280.
FIG. 12*a*.—Metastasis in ovary (Krukenberg) before inoculation. H. & E. × 280.
FIG. 12*b*.—Inoculated tumour in Fig. 12*a* after 15 days. Note mitotic figures. H. & E. × 280.
FIG. 13*a*.—Peritoneal metastasis from carcinoma of ovary before inoculation. H. & E. × 320.
FIG. 13*b*.—Inoculated tumour from Fig. 13*a* after 10 days. H. & E. × 320.
FIG. 14*a*.—Peritoneal metastasis from carcinoma of stomach before inoculation. H. & E. × 280.
FIG. 14*b*.—Inoculated tumour from Fig. 14*a* after 21 days. H. & E. × 280.
FIG. 15*a*.—Fibrosarcoma before inoculation. H. & E. × 280.
FIG. 15*b*.—Inoculated tumour in Fig. 15*a* after 10 days. H. & E. × 120.
FIG. 16.—Squamous cell carcinoma proliferating over surface of ventricle 21 days after inoculation H. & E. × 350.

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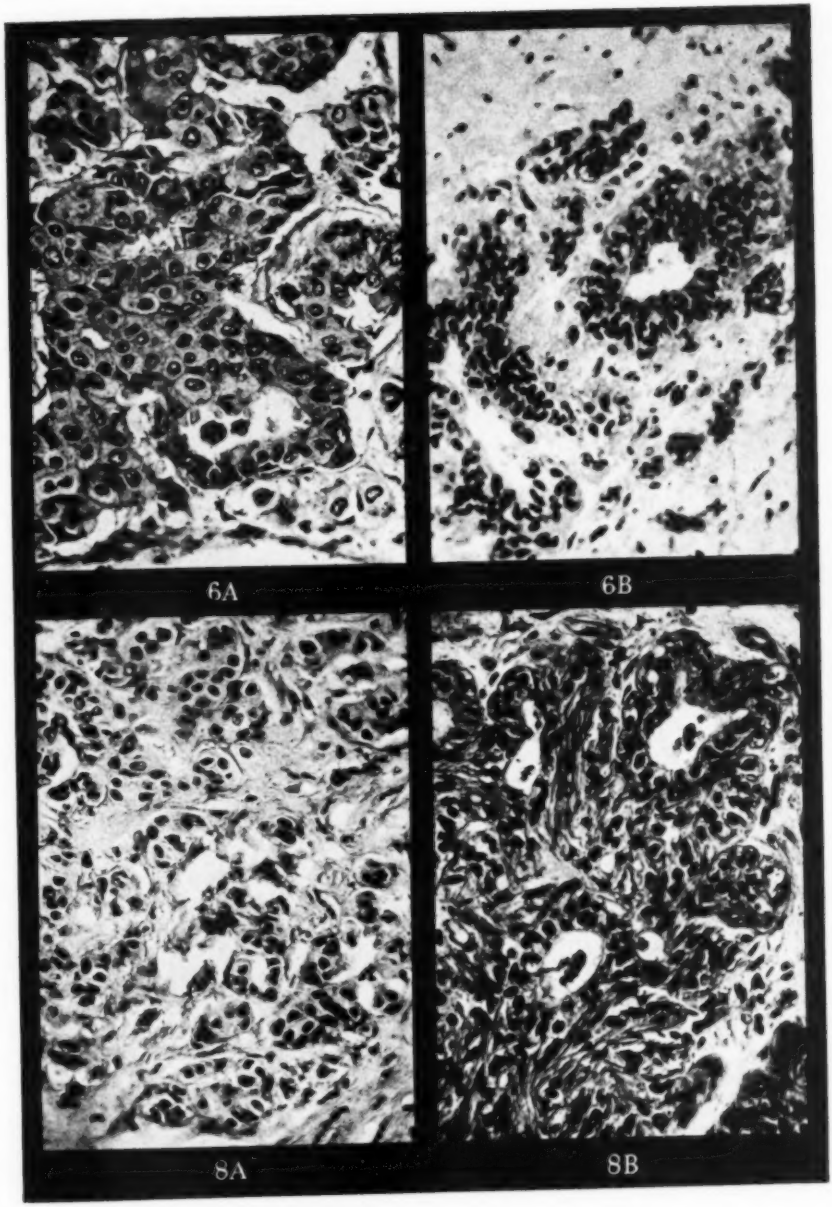


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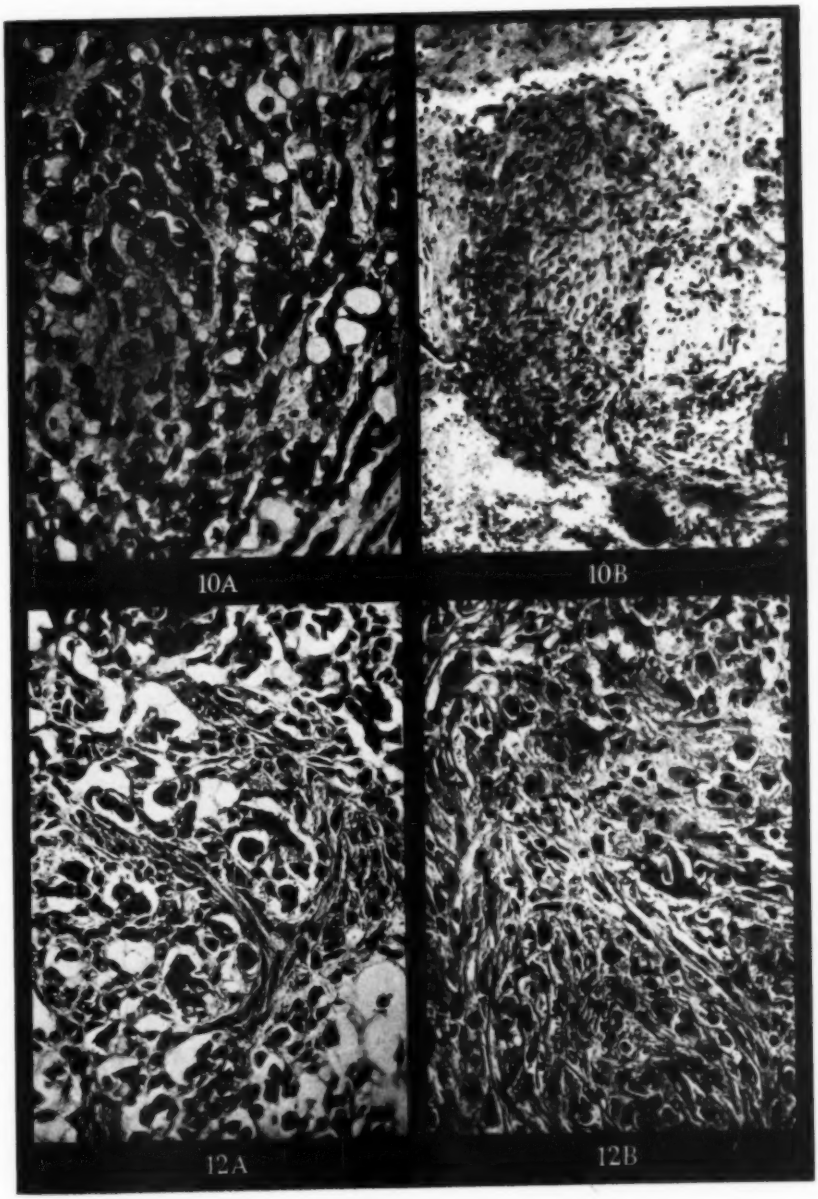
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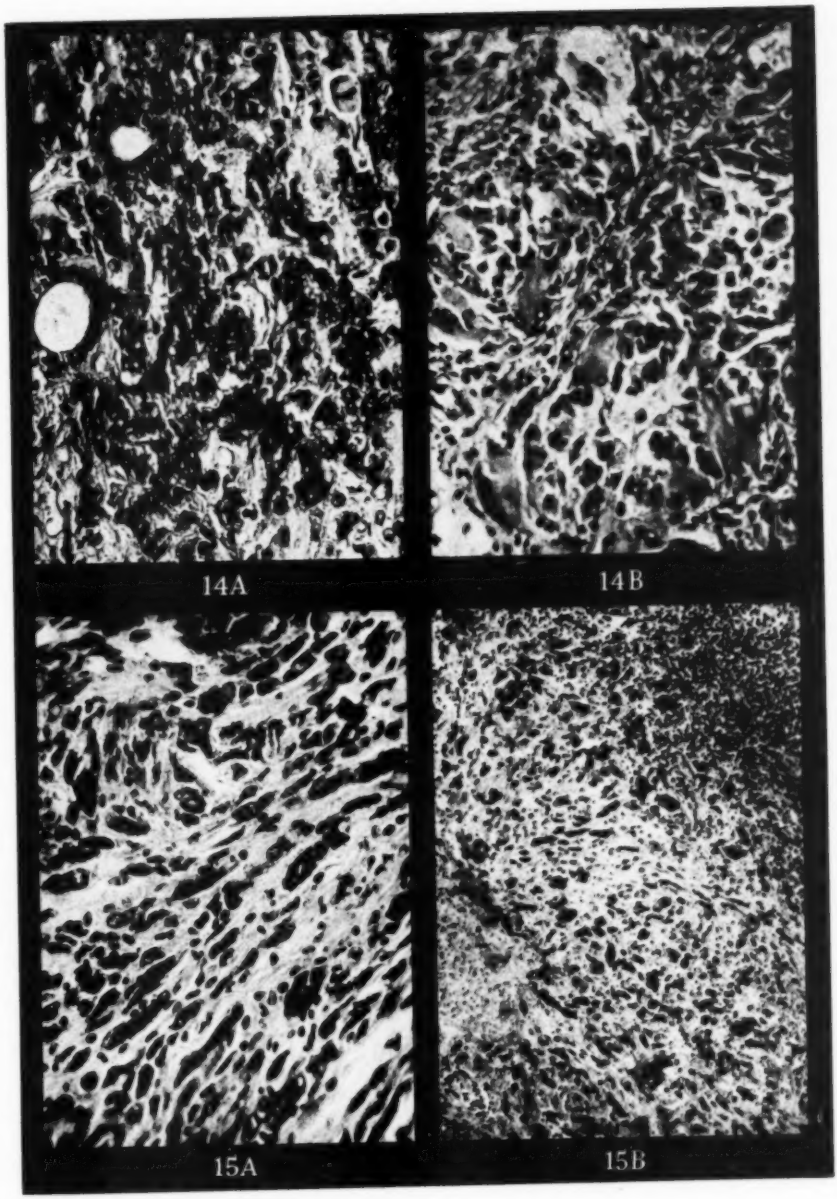

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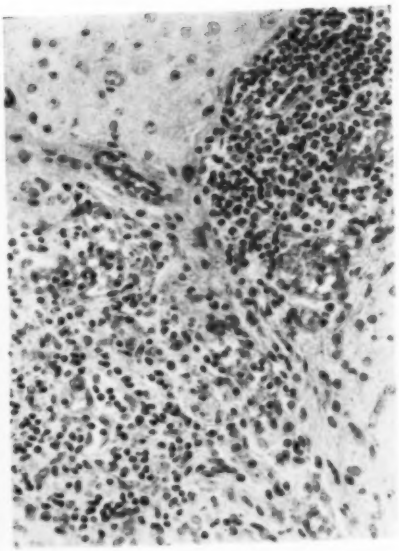
3



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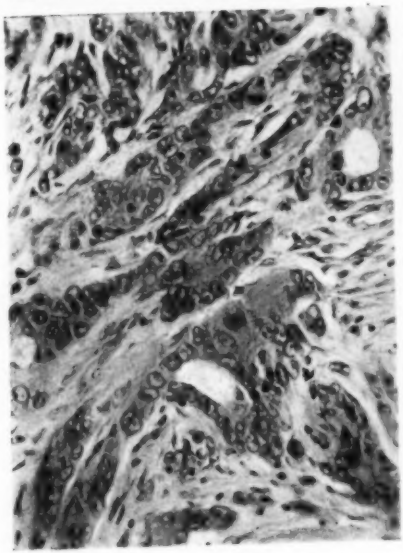
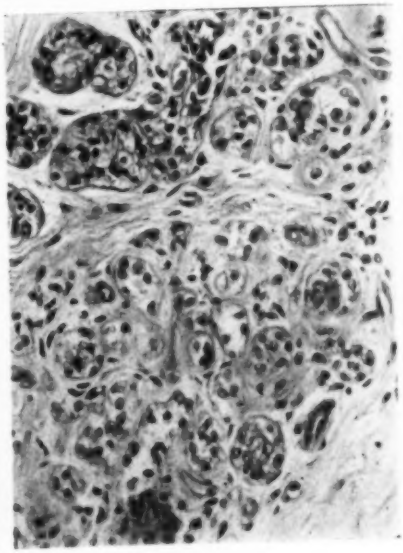
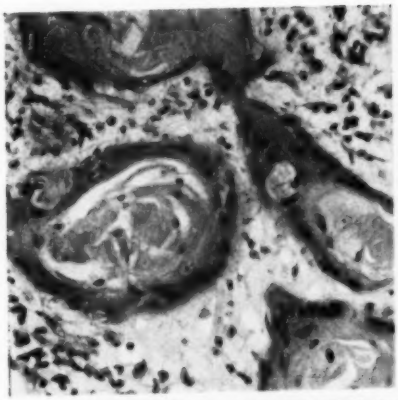




7b

9a



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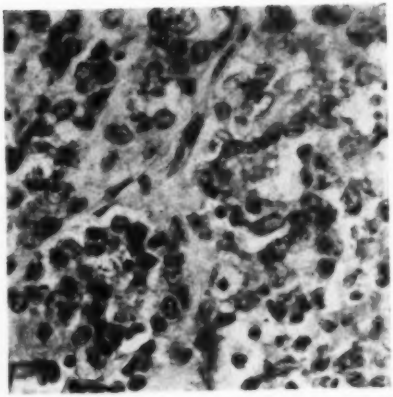

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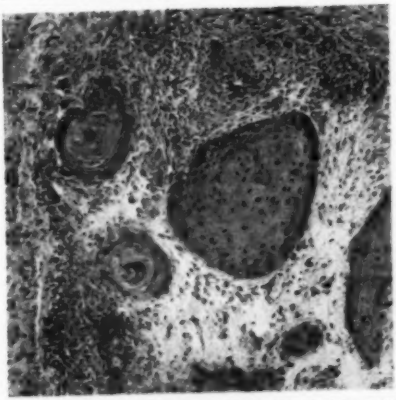

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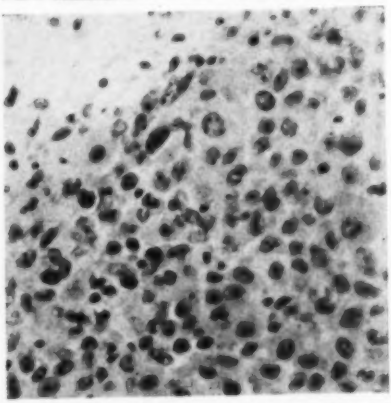

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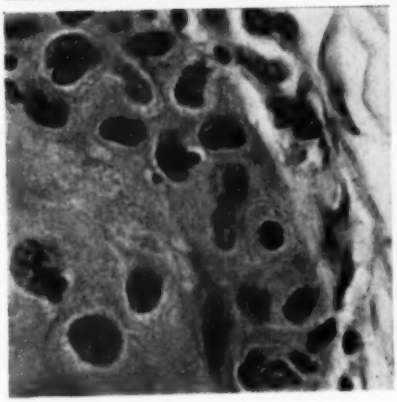

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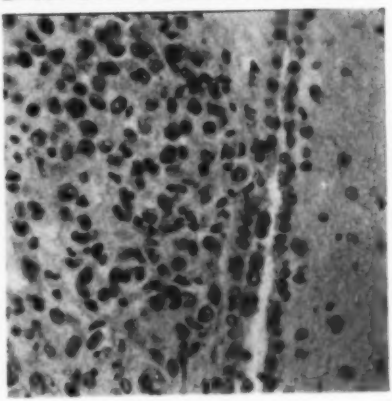
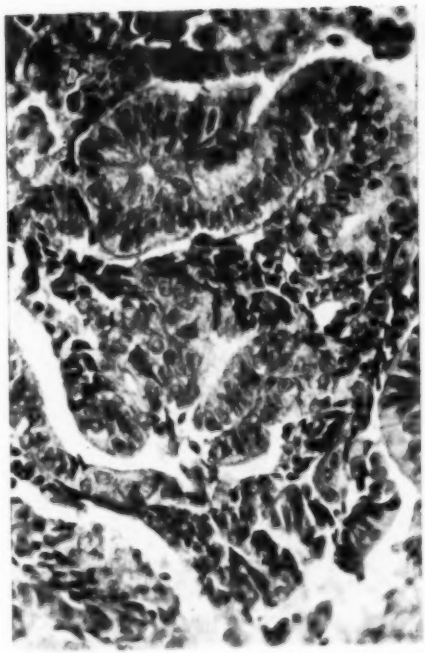
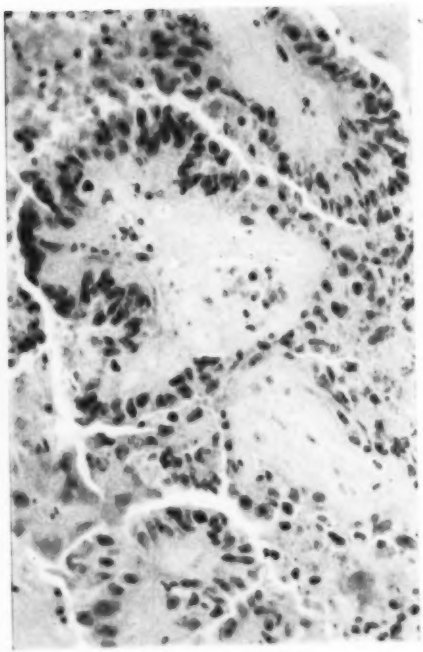


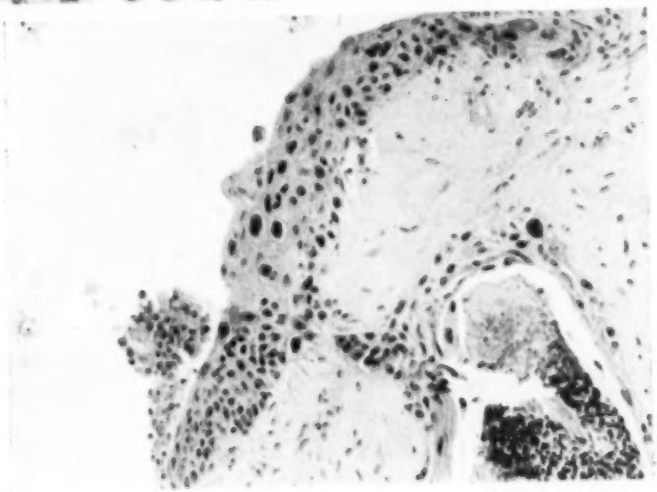
Lumb.

13*a*





13*b*



16

tumour cells have disappeared but when they are present they appear as clumps of cells unassociated with any cellular infiltration.

At the 21 and 28 day period vascularisation of the inoculation zone is the most striking feature. Small capillary blood vessels are seen in abundance and in the areas where tumour cells have disappeared fibroblast proliferation occurs (Fig. 5). Lymphocytic infiltration is not a marked feature of the cerebral inoculations and is restricted to the periphery of the tumour zone. This is in marked contradistinction to the appearances in the anterior chamber of guinea-pig or rabbit eye. In those cases where all tumour cells have been removed, a small focus of fibrous tissue is left from which all vessels gradually disappear leaving a small zone of organised collagen.

The clearly demarcated inoculation site in the brain is always very much larger than the tiny fragment of tumour tissue which is introduced. This zone is not always completely occupied by tumour cells and on many occasions the apparently large size of the site is the result of swelling by tissue fluid.

Certain special features have emerged in relation to specific tumours when introduced into the brain. In the case of breast carcinomas the tissue remaining in the transplantation area when the brains are examined tends to show clumps of well differentiated duct and acinar elements (Fig. 6*b*, 7*b* and 8*b*). At first sight some of these give the appearance of normal duct tissue, but so far no example of normal tissue surviving in the brain has been demonstrable. The explanation would appear to be either that the best differentiated areas survive or that in the new surroundings the cells tend to arrange themselves in patterns which are apparently well differentiated.

Breast tumours are probably the commonest malignant tumour available for transplantation purposes in that they usually occur free from bacterial contamination. They are unfortunately however, not the best suited tumours for this purpose as they tend to occur in their commonest form in close association with fibrous tissue. Twenty-seven have been used in this series and in 16 cases clumps of tumour cells have been found with the appearances described above. Squamous cell carcinomas have reproduced their normal histological appearances very closely in the mouse brain, producing easily recognisable " cell nests " (Fig. 9*b*) showing mitotic figures around the periphery of the cell clumps (Fig. 9*c*).

Malignant melanomas have also produced encouraging results where solid clumps of apparently viable cells with occasional mitoses have been found (Fig. 10*b* and 11*b*) and in one case evidence of what appears to be infiltration into the ventricle is seen (Fig. 11*c*).

Difficulty has been experienced so far with tumours of the gastro-intestinal tract because of bacterial contamination. Two cases of carcinoma of the rectum produced abscesses in the brain on inoculation. In 2 cases of carcinoma of the colon and a case of carcinoma of the stomach metastases were used with some success (Fig. 14*a*, and 14*b*).

Murphy (1926) in reporting his results of transplantation into mouse-brain stressed the fact that when tumours came in contact with the ventricle, rapid death resulted and he recommended that care should be taken to avoid inoculations near these structures. This has not been the experience in these experiments, for in a number of cases foci of tumour cells have been found in close association with the ventricles and in the case mentioned above, a melanoma appears to be directly infiltrating into a small narrowing of one (Fig. 11*c*). One squamous

cell carcinoma spread over the surface of the ventricle (Fig. 16) giving the impression that in this particular tumour the ventricular surface provided a particularly suitable medium for tumour cell proliferation.

The results which have been described so far are in relation to normal mice, but twenty of the human tumours were inoculated both into untreated mice and into mice previously treated by a single whole body dose of 500 r delivered by a 220 Kv. generator. This dose was found by a series of experiments to lower the peripheral leucocyte count from an average of 8,000–9,000 per cu. mm. to 400–500 per cu. mm. With this dose it was decided that a maximal fall of circulating leucocytes could be achieved compatible with absence of untoward effects in the mice. The purpose of treating the mice with pre-operative X-irradiation was to investigate whether or not a diminution of circulating leucocytes would affect transplantation results. Many authors, among whom Murphy (1926) in particular may be quoted, have suggested the importance of the role of the lymphocyte in heterologous transplantation.

The results obtained in this experiment showed no striking variations from those which were achieved in non-irradiated mice. It was decided that any difference did not merit a continuation of the experiment as it had been designed, for the following reason.

The maximum fall of circulating leucocytes occurs 48–60 hours after treatment and remains at the low level for about 7–10 days, when, if the mouse is unaffected, the level rises to normal again. It has been stated already that unlike the anterior chamber of the eye the lymphocytic response in the brain is minimal in the early stages after transplantation, and therefore it would seem likely that at the 3–4 week period when a depressed number of lymphocytes might be an advantage, very little effect would be gained from irradiation. It is felt that irradiation after the tumour has been inoculated would clearly be disadvantageous as it would tend to affect the transplanted tumour cells. For these reasons, therefore, irradiation has been discontinued except in those cases where serial transplantation is being studied.

## DISCUSSION.

In reporting results so far, care has been taken simply to record presence or absence of histologically recognisable tumour tissue without making any attempt to interpret results in terms of " takes." Many authors, including Snell (1954) and Towbin (1951*a*, 1951*b*), have stressed the confusion which arises by recording results simply in terms of " take " or " failure " without making clear the statement. It is obvious that if a transplanted tumour grew as a solid mass to destroy a considerable area of host tissue then no doubt would remain that frank growth was taking place in the transplanted area. The most striking evidence of success of transplantation would be metastasis within the new host, an occurrence which seems unlikely to happen and which has not been claimed even by workers whose results have been most successful.

So far in these experiments no example of metastasis or massive growth has been seen. Only occasionally are mitoses demonstrable and only in 2 cases has there been evidence of infiltration by tumour cells at the edge of the inoculation area. The appearances in " successful " cases have been of isolated areas at the transplantation sites sharply demarcated from adjacent brain showing clumps of cells which by the normal methods of histology appear to be viable. If it were

claimed that these cells represent simply a process of suspended animation somewhere between living and dying, but incapable of true proliferation in the manner of a tumour, then very little argument could be brought to the contrary. In the assessment of results so far, however, when careful consideration has been taken of the amount of apparently viable tumour material remaining demonstrable in the period up to 5 weeks after inoculation, the opinion has been formed that the most anaplastic tumours and in particular frank metastases from tumours produce the most active results (Table II).

TABLE II.

| Type of tumour. | No. cases inoculated. | No. cases with cells demonstrable in mouse-brain. | No. of failures. | Notes. |
|---|---|---|---|---|
| Carcinoma of breast | 27 | 16 | 11 | 8 failures were tumours which had not metastasised. 12 successes had already metastasised. |
| Squamous cell carcinoma | 8 | 8 | 1 | Six of the eight cases were metastases. |
| Melanoma | 6 | 4 | 2 | The two failures were primary. The four successes were metastases. |
| Carcinoma of ovary | 6 | 5 | 1 | Four of the five successes were metastases. The failure was a primary. |
| Carcinoma of stomach | 2 | 1 | 1 | |
| Carcinoma of rectum | 3 | 1 | 2 | Both failures were infected primaries. Success was a metastasis. |
| Carcinoma of colon | 3 | 3 | — | All cases had metastasised. |
| Carcinoma of lung | 1 | — | 1 | |
| Carcinoma of kidney | 1 | 1 | — | This was a very anaplastic primary tumour. |
| Sarcomas | 3 | 2 | 1 | The two successes were primaries. |

It has already been stated that Greene has claimed that heterologous transplantation results can be used as an assessment of the virulence of the human tumour in terms of the patient's progress and prognosis. No such immediate claim can be made as a result of these early experiments, but on the other hand it is felt that sufficient promise exists to make worth while a more extensive series of inoculations when the following facts must be established. In the first place it must be made certain whether all normal and benign material fails to survive a transplantation as our early findings and those of other workers suggest. Next, further malignant tumours must be studied in order to establish whether the apparent truth that the degree of malignancy as expressed by behaviour on transplantation can be substantiated.

If the apparent constancy of these results over a relatively small group of cases can be confirmed in large series, then the appearances seen in the trans-

plantation area could conceivably be used in the assessment of a biological test of degree of malignancy, irrespective of whether they represent the true power of growth proliferation or only of suspended animation or nidation.

SUMMARY.

1. A report is given of the results obtained following heterologous transplantation of sixty different human tumours into mouse-brain.

2. The histological appearances during the first 28 to 35 days after inoculation are described.

3. The merits of pre-operative irradiation of the mice are discussed.

4. The tentative suggestion is made that better results are obtained with more rapidly growing and metastasing tumours.

5. Necessity for rapidity of transfer from host to experimental animal is stressed.

This work has been made possible by a grant from the Imperial Cancer Research Fund. My grateful thanks are due to Professor Geoffrey Hadfield for much helpful advice and criticism, to Mr. J. M. Tancock for most able and painstaking assistance with the technical work, and to Mr. E. V. Willmott for the photomicrographs.

## REFERENCES.

DOBYNS, B. M., AND LENNON, B.—(1952) *Cancer*, **5**, 45.
GREENE, H. S. N.—(1938) *Science*, **88**, 357.—(1941) *J. exp. Med.*, **73**, 461, 475.—(1942) *Cancer Res.*, **2**, 649.—(1943) *Ibid.*, **3**, 809.—(1950) *Yale J. Biol. Med.*, **22**, 611.—(1951) *Cancer Res.*, **11**, 529.—(1952) *Cancer*, **5**, 24.
*Idem* AND LUND, P. K.—(1944) *Cancer Res.*, **4**, 352.
*Idem* AND MURPHY, E. D.—(1945) *Ibid.*, **5**, 269.
LEMON, H. M., AND PARSONS, L.—(1952) *Boston med. Quart.*, **3**, 39.
LOEB, L.—(1930) *Phys. Rev.*, **10**, 547.
MORRIS, D. S., MCDONALD, J. R., AND MANN, F. C.—(1950) *Cancer Res.*, **10**, 36.
MURPHY, J. B.—(1926) *Monogr. Rockefeller Inst. med. Res.*, **21**, 1.
SHIRAI, Y.—(1921) *Japan med. World*, **1**, 14.
SMIRNOVA, E.—(1937) *Bull. Biol. Méd. exp. URSS.*, **4**, 6.
SNELL, G. D.—(1954) 'The Physiopathology of Cancer.' London (Homburger and Fishman), p. 338.
TOOLAN, H. W.—(1951) *Proc. Soc. exp. Biol., N.Y.*, **77**, 572.
TOWBIN, A.—(1951*a*) *Cancer Res.*, **11**, 76.—(1951*b*) *Arch. Path.*, **52**, 199.
VAN DOOREMAAL, J. C.—(1873) *V. Graefes. Arch. Ophthal.*, **19**, 359.
ZAHN, F. W.—(1884) *Virchows. Arch.*, **95**, 369.

# CARCINOMA AND ATROPHY INDUCED BY LOCAL SURFACE X-IRRADIATION IN THE MAMMARY GLAND IN RIIIb MICE.

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HYPERPLASIA, atrophy, chronic inflammation and scarring have all been described as antecedents of spontaneous cancer. More often no previous pathological change has been detected. Of the known antecedents, atrophy is the most surprising because it is a retrogression believed to be irreversible. The association of cancer combined with atrophy thus raises the question of the order of events. Did neoplastic change occur before or after observed atrophy? It ought to be possible to devise experiments to decide whether new growth can be initiated and imposed in cells already in a state of atrophy or whether this change, if it occurs, precedes the atrophy. In experimental pathology the antecedent of cancer most commonly observed is hyperplasia. Prolonged hyperplasia is sometimes succeeded by atrophy and tumour growth. A nutritional atrophy combined with fatty infiltration due to choline deficiency is associated with hepatoma in rats but in neither circumstance is the order of events known relative to actual initiation of new growth. The term " initiation " is used in the sense introduced by Friedewald and Rous (1944). Experiments designed to decide the order of events would be done in two stages: first an endeavour to bring about true atrophy using for the purpose some non-carcinogenic agent or method, and second the application of a specific carcinogen to the region made atrophic. Attempts by the author to carry out this sequence revealed a constant tendency of mouse skin—the most suitable tissue to use—to undergo hyperplasia in response to all varieties of injury that were tried. Hair follicles may be destroyed, for example by repeated excision of the same area of skin, giving an appearance of local atrophy, but the epidermis itself always thickens. A trial was next made with an agent known from human pathology to cause both atrophy and new growth in the expectation that the order of events might be deduced by the difference in dosage required for the two effects.

The experiments here recorded made use of mammary epithelium and repeated X-irradiation. By means of prolonged whole body irradiation with radium Lorenz, Eschenbrenner, Heston and Uphoff (1951) increased the prevalence and decreased the time of appearance of mammary tumours in C3Hb mice without milk-factor but attributed these tumours in part to stimulation of the mammary gland by secretion from granulosa-celled tumours of the ovary which were due to and arose during the course of irradiation. To avoid this possible additional factor, local surface X-irradiation of 2nd and 3rd right nipple regions, which overlap, was carried out. Five palpable mammary carcinomas and four which were visible during microdissection and after bulk-staining arose in 27 virgin female

mice of the RIIIb strain, free of milk-factor, which lived for a sufficient period of time. The small tumours found during the course of microdissection at 7× magnification enabled one to see their site of origin. The nature of two of these was confirmed by section. An increase in benign mammary tumours and several examples of atypical intraduct proliferation were also seen. No spontaneous mammary carcinoma was found in 60 long-lived unmated females of this strain. Less than 2 per cent have occurred in breeders. All the treated nipple regions became atrophic but the order of events could not be determined. Nevertheless the results are here recorded because mammary carcinomas were induced without ovarian tumours ; no skin tumours arose in the overlying epidermis ; the problem itself appears to be worth attention.

## METHODS.

### *Preparation of mice.*

Young RIIIb virgin females of 3 to 5 months old without milk-factor were epilated with barium sulphide over an area a little more than 1·5 cm. in diameter in the right axillae and just including the 2nd and 3rd nipples. Hair grew again after irradiation but scantily and required snipping only very occasionally. In order to keep the mice still in position during local irradiation each was given a dose of 0·3 ml. of a 3 per cent suspension of bromethol subcutaneously. When anaesthetised the mice were lightly strapped in position with adhesive plaster on a cork board so that the second and third nipple regions lay uppermost. The right foreleg was fixed on the right side beyond the shoulder.

### *Irradiation.*

Local irradiation was carried out with a Victor X-ray unit using 85 kV, 5 m.a. with 1 mm. Al. filter. An applicator of 1·5 cm. diameter was placed over and just in contact with axillary and thoracic skin so as to include within the areas irradiated the second and third nipples on the right side. The dose rate was 101 roentgens per minute. A dose of 600 r was given once per week. Various total doses from 3000 r to 6000 r were used. About $\frac{1}{8}$ of the dose as found by measurement penetrated to the outside of the skin on the left side thus passing through the 2nd and 3rd *left* nipple regions. Owing to the position of the mice the interscapular lobe of the second nipple area was further distant from the applicator than the rest of the treated site. Since the interscapular lobes meet in the middorsal line, the dose to the two lobes at this site was about equal and somewhere between a third and the full dose.

### *Examination of mammae.*

All nipple areas were examined with a binocular dissecting microscope unless the mice had died unexpectedly and decomposition was too advanced for study. Three such mice have been omitted from the final count of survivors beyond 11½ months old, one for each of the doses used, although it can be stated that they did not have palpable tumours. Results have been based on examination with the dissecting microscope and it would have been impossible to exclude the presence of small growths in such decomposed and partly eaten animals. Right and left nipple areas were dissected out and fixed separately in Bouin's fixative. All were stained in bulk and examined as previously described (Pullinger, 1947,

1949). As a rule 1st, 2nd and 3rd nipple areas of both sides were mounted and kept. The 4th and 5th, which are bulky, were preserved only if they differed materially from the 1st. The 1st, 4th and 5th pairs of nipple areas served as indicators of circulating ovarian hormone. The irradiated mammary epithelium might have responded similarly to this hormone but in the event did not owing to vascular damage or to atrophy, or both. Microscopic sections were made of all but 2 tumours and a few were grafted.

## RESULTS.

Results relating to tumour incidence are summarised in Table I.

TABLE I.—*Mammary Tumours in Unmated RIIIb Mice after Local Surface and Penetrating X-irradiation of Second and Third Nipple Regions.*

| No. of experiment. | Total dose in r. | Number of mice. | | | |
|---|---|---|---|---|---|
| | | Treated. | Alive at 11½ months. | With carinoma. | With benign growths. |
| II . . | 6000 . | 14 | 6 | 1 | 0 |
| III . . | 4800 . | 12 | 10 | 4 | 5 |
| IV and V . | 3000 . | 23 | 11 | 4* | 6 |
| Control . . | None . | None | 21 | 0 | 1 |
| Control . . | None . | None | 33 | 0 | 5 |

* Includes one which died at 8 months old.

Experiment I, which is not included here, was done to determine the dose level.

Experiment II : 6000 r in 10 weekly doses of 600 r was given to 14 mice aged 3 months. One mouse, No. 10, killed at 20 months old, had a mammary carcinoma. The 2nd right nipple area contained a small extraductal growth and also some intraduct growth near the nipple (Fig. 1). A small rectangular piece of tumour was cut out for section and proved to be an alveolar carcinoma. Two sarcomas had developed in another mouse by 9½ months of age.

Experiment III : 4800 r in 8 weekly doses of 600 r was given to 12 mice aged 4 to 4½ months. Mice numbered 4, 10, 11 and 12 developed mammary tumours and number 10 a sarcoma as well.

Mouse 4 was killed aged 14½ months. A slowly growing keratinising squamous-celled carcinoma was palpable in the 2nd right interscapular nipple area. Grafts from it survived in RIIIb males for 8 months. When examined only one had increased appreciably in size, though all had vascularised and were slightly larger.

Mouse 10 was killed aged 20½ months. A large polymorphic-celled sarcoma was found in the right side of the thorax. In the interscapular lobe of the 2nd *left* nipple area there was a small palpable tumour which proved to be an alveolar carcinoma. A main duct of the same nipple area was filled with growth, also several smaller ducts at its anterior border. Sections of these showed early intraduct growth which had arisen *in situ* and penetrated the duct wall in places (Fig. 2).

Mouse 11 was killed aged 12½ months. The 2nd right nipple area contained a rapidly growing alveolar carcinoma in the axilla, approximately 1 × 1½ cm. It was grafted and grew in 6 out of 6 RIIIb males.

Mouse 12 died aged 16½ months. A small carcinoma consisting of intra- and extra-duct growth was found by dissection in the 2nd right nipple area. Acinus formation could be seen at 100× magnification within one of the solid ducts of the whole mount (Fig. 3).

Experiment IV : 3000 r in 5 weekly doses of 600 r was given to 14 mice aged 4 to 4½ months old.

Mouse 22 died aged 8 months in an epidemic due to *B. piliformis* which killed all but 2 of this group. In the 2nd right nipple area in the isthmus between the proximal and distal interscapular lobes, there were 2 large nodules of acinar proliferation, one of which was dense and spherical resembling a small carcinoma rather than a benign tumour. The other of the two might have been either benign or malignant (Fig. 4).

Experiment V was done to supplement losses in IV. 3000 r in 5 weekly doses of 600 r was given to 9 mice aged 4½ to 5 months. Mice numbers 27, 30, and 33 developed mammary tumours.

Mouse 27 was killed aged 22 months. A small tumour was revealed by microdissection in the isthmus of the 2nd *left* nipple area (that is between the interscapular, axillary and proximal lobes). Sections of a dense portion of the tumour confirmed that it was an adenoacanthoma. This nipple area had received a dose of approximately 1000 r.

Mouse 30 was killed aged 20 months. There was a palpable mammary tumour in the mid-interscapular line. On dissection the greater part of it lay on the left of the mid-line having pushed aside the ducts of the 2nd left nipple area. The growth had arisen on the right side. It was an adenoacarcinoma about 0·6 cm. in diameter.

Mouse 33 was killed aged 11½ months. There was a palpable tumour in the 2nd right nipple region in the isthmus and distal interscapular lobe. It was an adenoacanthoma about 1 cm. in diameter.

Besides these malignant tumours there was an increase of apparently benign mammary growths in irradiated animals as compared with untreated controls (Table I). There were also many much smaller acinar and intraduct proliferations. The latter have only rarely been found in untreated mice whether unmated or breeders. The relationship of three of the actual tumours to ducts suggests the possibility of an origin from them. The majority of intraduct proliferations and benign growths, like the majority, 7 out of 9, of malignant tumours occurred on the right side exposed to maximum irradiation. For the doses 3000 r and 4800 r the maximum appeared to be the most effective for tumour induction. Losses in the experiment using 6000 r were too great to judge the effectiveness of this dose and are attributed to the younger age of the mice used and possibly too high a dose.

Three sarcomas occurred in 2 mice. There was no increase in lymphomas and no skin tumours arose. A few mice developed linear ulcers in the treated skin on the right side in later months of Experiments II and III. The epidermis was atrophic in that hair was scanty but the greater part of the surface epithelium was hyperplastic in section. Glücksmann (1951) together with Boag produced a few skin tumours by local treatment of skin in C57 black mice with a single exposure to an electron beam and dose of 7900 rep.

No ovarian tumours were found. The ovaries became atrophic but oestrus cycles were maintained in all those examined at a year old.

The mammae of 17 mice derived from all experiments were examined in early stages before the appearance of the first tumours. Atrophy was found in right second and third nipple areas by the fourth month after irradiation had been started. It was judged by reduction in lateral branching at first combined with shrinkage of the nipple area later. It was found in all of 11 mice examined at 5 months old. Involution, with which atrophy might be confused, does not begin to appear in unmated females of this strain until 10 to 11 months old. In addition 12 mice were killed for observation after 2 irradiations of 600 r each, 2 weeks after the first. By that time the mice were $4\frac{1}{2}$ to 5 months old. The treated right nipple areas were either unchanged or showed some degree of atrophy. At no time was there any hyperplasia beyond the degree of lobular alveolar development which is characteristic of at least half of the normal young unmated females of this strain.

During the course of irradiation vascular damage was done in the lungs but recovery took place. The treated nipple regions on the right side were more firmly adherent to subcutaneous tissue and to one another than normally and in consequence were less readily dissected without damage. A slight contraction of the right side of the thorax was noticeable. At one year old the weight of survivors in Experiment II was the same as in untreated controls.

## COMMENT.

It is clear that carcinoma arose from acinar and intraduct epithelium as a result of X-irradiation in the absence of ovarian tumours or of mammary hyperplasia. The new growths were accompanied by atrophy which began to appear earlier than 5 months old and was found in all mice by 8 months. Atrophy was probably due to some degree of vascular damage short of complete occlusion because the ducts persisted. In that case circulating oestrogen which was sufficient to keep up oestrus cycles and to maintain untreated nipple areas in a normal state could have reached the atrophic regions. Yet there was no response to be seen in them indicating that the atrophy was irreversible. Eight of the 9 carcinomas were found in mice older that 8 months so that it can be said that the conditions responsible for atrophy of neighbouring cells did not prevent subsequent growth, an observation which is in accord with human and experimental pathology. No conclusion can be drawn concerning the time or state of the mammae when the tumours were actually initiated. One of the tumours (on the left side) occurred after exposure to approximately 1000 r while atrophy had not begun in all mice after 1200 r. The necessity for a two stage experiment with two different agents is again apparent.

## SUMMARY.

1. Mammary carcinoma was induced in 9 out of 27 unmated RIIIb female mice without milk factor by local surface and penetrating X-irradiation of second and third nipple areas. The growths arose from intraduct and acinar epithelium. No spontaneous carcinoma occurred in unmated females of this strain. Less than 2 per cent were found in breeders.

2. Growth of tumours was preceded by atrophy but the sequence of events concerning the time of onset or initiation of actual neoplastic change could not be ascertained. The atrophic process did not prevent subsequent growth of the tumours. Hyperplasia was not seen at any stage.

3. No ovarian or skin tumours arose, nor was there any increase in lung adenoma or in lymphoma. There were 3 sarcomas.

The standardised nomenclature of the mouse strain used is RIIIfB/Pu.

I am greatly indebted to Dr. P. R. Peacock, the Board of Management of the Royal Beatson Memorial Hospital, and to Dr. J. M. Glennie for generous allocation of space and facilities to do these experiments.

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## REFERENCES.

FRIEDEWALD, W. F., AND ROUS, P.—(1944) *J. exp. Med.*, **80**, 101.

GLÜCKSMANN, A.—(1951) *J. Path. Bact.*, **63**, 176.

LORENZ, E., ESCHENBRENNER, A. B., HESTON, W. E., AND UPHOFF, D.—(1951) *J. nat. Cancer Inst.*, **11**, 947.

PULLINGER, B. D.—(1947) *Brit. J. Cancer*, **1**, 177.—(1949) *Ibid.*, **3**, 494.

## EXPLANATION OF PLATES.

FIG. 1.—Experiment II, Mouse 10. Right (*a*) and left (*b*) second nipple areas. The right contained a small extra- and intraduct carcinoma marked 1. Atrophy and shrinkage are conspicuous in the right. Approximate position of left nipple marked N. × 3.

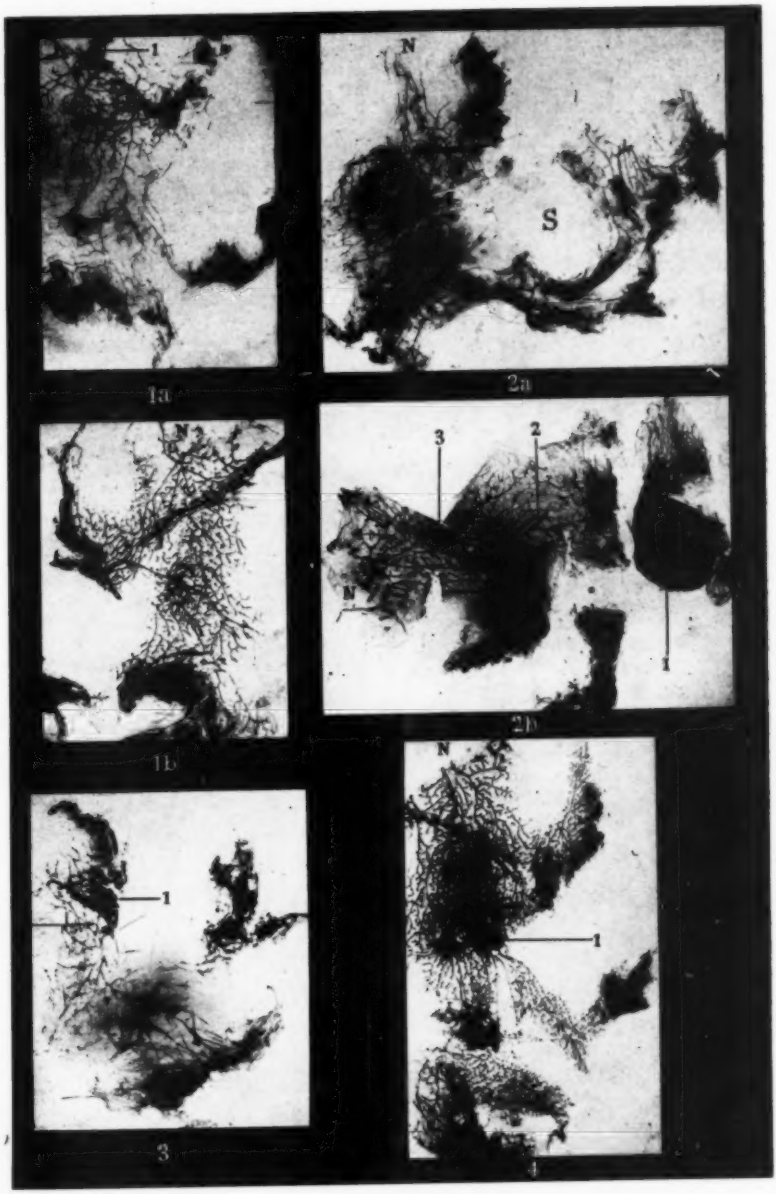
FIG. 2.—Experiment III. Mouse 10. (*a*) Right second and third nipple areas have been pushed apart by a sarcoma found in position 8. The second right nipple was near N. (*b*) The second left nipple region contained a carcinoma 1 in the interscapular lobe, intraduct growth in a main duct 2 and in ducts cut out from 3. Atrophy is conspicuous on both sides except around the left nipple N. × 3.

FIG. 3.—Experiment III, Mouse 12. Right second nipple area. A small extra- and intraduct carcinoma is shown at 1. Intraduct acinus formation could be seen at 100 × magnification. × 3.

FIG. 4.—Experiment IV, Mouse 22. Right second nipple area. A small adeno-carcinoma is shown at 1. Atrophy had begun by 8 months. Lateral branching and lobular-alveolar differentiation of the other nipple areas were much more developed. × 3.

The unmarked black areas are masses of fat in which the gland is embedded.

Fig. 1, left nipple area is indistinguishable from a normal involuting second nipple area of the same age.




Pullinger.

# THE HISTOGENESIS OF STILBOESTROL-INDUCED RENAL TUMOURS IN THE MALE GOLDEN HAMSTER.

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SEVERAL observers have shown (Pfeiffer, Emmel and Gardner, 1940; Korenchevsky and Ross, 1940) that apart from the occasional production of urinary calculi and glomerulo-nephritis, prolonged oestrogen administration has only a slight effect on the kidneys of rodents, and never induces neoplasia. Matthews, Kirkman and Bacon (1947) were the first to demonstrate that the kidneys of the male golden hamster (Cricetus auratus) are the exception as they possess a peculiar susceptibility to renal neoplasia following treatment with oestrogen.

The object of this communication is primarily to describe the histogenesis of these oestrogen-induced renal tumours in the male hamster, and to discuss other related problems which might help towards a better understanding of oestrogenic neoplasia.

## MATERIAL AND METHODS.

Forty male golden hamsters, all approximately 12 weeks of age, each received a 20 mg. pellet of pure diethylstilboestrol subcutaneously in their right flank. An equal number of untreated male hamsters of the same group were kept as controls.

For routine histological study the kidneys were fixed in alcoholic Bouin, and subsequently stained either in haematoxylin and eosin or by a modification of Masson's light green. In order to test for the presence of intracellular lipids, both fresh and formalin-fixed kidneys were stained with alkaline Sudan IV, Sudan black B and Nile blue sulphate. The pituitaries were fixed in Zenker-formol and differentially stained by Mallory's triple stain. Verhoeff's method was used for the staining of elastic fibres and the Gordon and Sweet procedure for the presence of reticulin.

## OBSERVATIONS.

### *Macroscopic description of renal tumours.*

Six and a half months after stilboestrol treatment had commenced all hamsters were palpated weekly for the presence of kidney lesions.

Although no renal tumours were palpable at this early period of treatment 8 hamsters were killed in order to study the development of early tumour formation. Each of these hamsters possessed very small cortical lesions which varied between 2 and 5 mm. in diameter. Small palpable tumours arose in some hamsters at the end of the 9th month of treatment, and by the beginning of the 11th month every treated hamster had large renal tumours. At the end of the 12th month

all the hamsters belonging to the untreated control group were killed and none was found to have developed renal tumours.

In every instance these kidney tumours were bilateral and multiple, and in their early stages, cortical in position. They were distributed through all levels of the renal cortex and those which were subcapsular in position projected from the surface of the kidney (Fig. 2). Involvement of the renal medulla sometimes occurred but was due to extension of a tumour which was primarily within the cortex. In their early stages the tumours were compact and white but cysts, (Fig. 1) yellowish areas and haemorrhage were common in the larger tumours. Each had a well defined though non-encapsulated periphery.

In three instances large tumours have been associated with multiple tumour deposits of similar appearance within the peritoneal cavity. This appears to be the result of rupture of an anteriorly situated tumour and implantation of tumour cells on the peritoneum. Such metastatic nodules were present on the peritoneal surface of the diaphragm, body wall, liver, ascending colon, and mesentery in all 3 animals. There was no macroscopic evidence of secondary deposits either in the lymph nodes or lungs.

Twenty-eight of the hamsters treated with stilboestrol for 10 months or over developed pituitary tumours. Nine had enlarged prostate glands, 10 had a marked cystic dilatation of the epididymis and 3 developed an infection of their seminal vesicles. Six hamsters had enlarged adrenals, and with the exception of one case, the hypertrophy was unilateral.

*Microscopic description.*

Examination of the earliest tumour foci allows clear recognition of their origin from the epithelium of cortical tubules (Fig. 4) and in some instances it is possible to identify the involved tubules as of convoluted type. No instance of genesis from medullary tubules has so far been encountered. Most of the early foci are situated close to glomeruli and, though they are widely scattered through the cortex, subcapsular foci are especially numerous. In some hamsters there has been a striking involvement of tubules about arcuate vessels.

Involved tubules show first nuclear enlargement and loss of cytoplasmic eosinophilia of their epithelial cells. Mitoses appear and there is progressive cell-multiplication until the tubular lumen is obliterated by a compact mass of darker-staining, cuboidal, spheroidal or fusiform cells of indistinct cytoplasmic outline. Expansion and growth of the tubule is followed by rupture of its basement membrane and extension of the proliferating epithelial cells between adjacent tubules (Fig. 7). It is possible to recognise, at times, origin within a segment of a tubule or simultaneously within a few neighbouring tubules—a "field" of origin. Growth is not only by expansion but also by peripheral infiltration. Proof of infiltration, difficult to prove unequivocally within the renal substance where progressive involvement of adjacent tubules is an alternative explanation, is provided by those tumours situated at the kidney surface which extend in typical infiltrative manner into the peri-renal fat (Fig. 8). Compression of surrounding tubules sometimes produces a pseudo-capsule but true encapsulation by fibrosis does not occur. In the numerous kidneys examined, neither lymphatic nor blood-vascular invasion by tumour cells has been found.

Well established tumours consist of sheets and cords of compactly grouped cells of uniform appearance, solid acinar cell groups bounded by capillaries,

or pseudo-glandular structures produced by cubical or columnar cells arranged in palisade fashion about capillaries. A papillary arrangement (Fig. 5) occurs but tubular differentiation is infrequent. In some areas the tumour cells are of spindle shape, producing a sarcoma-like appearance (Fig. 6). A constant feature in all these growths is the appearance even at an early stage of a doubly refractile lipid within the cytoplasm of tumour cells. This lipid stains positively with alkaline Sudan IV (red), Sudan black B (black) and Nile blue sulphate (red) and gives a positive periodic acid-Schiff reaction which is unaltered by prior diastase digestion. Negative reactions were obtained with Best's method for glycogen, mucicarmine, Schmorl's thionin method for metachromasia, the method of McManus and Cason for prevention of the periodic acid-Schiff reaction by acetylation—indicating absence of 1 : 2 glycol groups, Millon's method for proteins containing tyrosine, Sakaguchi's method for arginine and the Ninhydrin-Schiff method for amino acids. In paraffin sections much of the lipid is lost, leaving "clear" cells (Fig. 9) but some remains as irregular eosinophil inclusions (Fig. 10). Cysts, many of which develop to a large size, are very common : most appear to arise from engulfed non-neoplastic tubules but others result from dilated neoplastic tubules or necrosis of tumour tissue. Haemorrhage, collections of pigment-containing histiocytes, necrosis and foci of calcification (Fig. 6) are found in the larger tumours. The structure of peritoneal deposits is similar to that of the renal tumours (Fig. 3).

*Associated oestrogenic changes in organs other than the kidney.*

Many of the hamsters which had developed renal tumours as the result of stilboestrol treatment, showed accompanying changes in the pituitary, adrenal and prostrate glands as well as in the epididymis, seminal vesicles and testes. The most pronounced changes were observed in the hypophyses. Twenty-eight out of the 40 treated male hamsters developed hypophyseal tumours. With the exception of five tumours, which were lesions involving the pars intermedia of the gland, they were all chromophobe adenomas of the pars anterior. In two cases the tumours which originated in the pars intermedia had invaded the pars posterior and the pars anterior of the gland. Both these lesions were actively growing tumours since mitoses were numerous (Fig. 11, 12, 13). The lesion in a third pituitary gland was a nodular hyperplasia of the pars intermedia without any invasive growth. The chromophobe adenomas were identical in structure with those previously produced by prolonged oestrogen administration in other kinds of rodents, Cramer and Horning (1936), Zondek (1936), Burrows (1936) and Deanesley (1939).

The abnormalities caused by oestrogen in the prostatic epithelium of the hamster are of unusual interest. In one hamster treated for 10½ months, several of the alveoli showed the usual squamous metaplasia accompanied by keratinisation (Fig. 15), so typical of the reaction of the rodent prostrate to the female sex hormone, whilst in adjacent alveoli the glandular epithelium had undergone a marked hyperplasia without any evidence of squamous changes (Fig. 14).

Hyperplastic foci developed in the adrenal cortex in 3 hamsters which had received stilboestrol treatment for 8 to 9 months. Cell-divisions were numerous (Fig. 16). All 3 of these hamsters had developed pituitary tumours of the pars anterior.

The effect of prolonged oestrogen administration on the epididymis was to

induce an atrophy of the epithelium accompanied by a loss of secretory activity and an increase of the fibromuscular stroma. In the more advanced cases a pronounced squamous metaplasia of the glandular epithelium developed in 5 of the treated hamsters. No tumours of the epididymis developed in any of the treated animals.

The testes in the treated hamsters showed a marked individual variation in sensitivity to oestrogen. In most instances the testes were atrophic and there was an inhibition of spermatogenesis accompanied in several instances by a hyperplasia of the interstitial tissue. Eleven hamsters treated for 10 months, all of which had developed chromophobe adenomas of the anterior hypophysis, showed only a slight degree of atrophy of the seminiferous tubules without any arrest of spermatogenesis.

The changes produced in the seminal vesicles by oestrinisation were also variable and were similar to those previously reported in other rodents, de Jongh (1934), Van Wagenen (1935) and Wells (1936).

## DISCUSSION.

The fact that no tumours developed in the kidneys of the control untreated hamsters leaves little doubt that the lesions in the treated animals arose as the result of excessive oestrogenic stimulation.

It is interesting that oestrogen administration induces renal tumours in the male but not in the female hamster, Kirkman and Bacon (1949). These results, however, fall into line with what is already known of the effect of sex on the responsiveness of tissues to oestrogen. Males appear to be more reactive than females to the female sex hormone. Burrows and Horning (1952) are of the opinion that the exposure of the female to plentiful amounts of oestrogen during life may possibly cause some degree of physiological adaptation. Lipschutz, Vargas and Palma (1941) have found that oestrogen-induced tumours of the sub-peritoneum of guinea-pigs can be produced in both males and females, but to produce fibrous tumours in the male the dosage needed was approximately 8 times that which would produce a comparable effect in the female guinea-pig. Experiments are already in progress to determine if renal neoplasia can be induced in the female hamster by employing doses of stilboestrol 5 times as strong as those previously used when treating the males.

It is difficult to understand why the kidney of the male hamster possesses this peculiar susceptibility to renal neoplasia following oestrogen administration whilst other kinds of rodents after similar treatment never develop kidney tumours. The reason why various animal species do not respond to oestrogen stimulation in the same way is not fully understood. Discrete fibroids, for instance, are readily produced throughout the subperitoneal tissue of the guinea-pig, but never arise in the hamster, rat or mouse after similar treatment.

These experiments on the induction of renal tumours in the male golden hamster by prolonged stilboestrol administration confirm the results previously obtained by Matthews, Kirkman and Bacon (1947), and Kirkman and Bacon (1949). Detailed studies of the histogenesis of these tumours reveal that they arise by progressive hyperplasia of the cortical tubular epithelium, rapidly enlarge and grow not only by expansion but also by peripheral infiltration. Rupture into the peritoneal cavity results in implant metastases on the abdominal parieties and viscera. No blood-borne metastases have so far been observed.

These renal tumours, judging from their histological structure and modes of extension, should be regarded as malignant growths (carcinomas). We find there is no structural distinction between the earliest hyperplastic stages and neoplasia, nor between a possible stage of benign neoplasia and malignancy. In this respect they resemble those renal cortical carcinomas of man which, unassociated with nephritic or arteriosclerotic scarring, probably arise without a preceding adenoma. Kirkman and Bacon (1950) however are of the opinion that the early subcapsular lesions are adenomatous in character. Structurally, the hamster tumours have in common with human renal cortical carcinomas the following features—the cystic, haemorrhagic and yellowish gross appearance of larger tumours and, histologically, "clear" cells, papillary structure, spindle-cell (sarcoma-like) areas and focal calcification. The intracellular doubly refractile lipid within the "clear" tumour cells is still under investigation. It would appear, from histochemical reactions, that the lipid is not associated or linked with protein and that its positive periodic acid-Schiff reaction is not due to the 1 : 2 glycol grouping. Human renal cortical carcinomas differ in that vacuolation of their cells is due not only to the presence of doubly refractile lipid but also to glycogen.

Of all the changes induced by oestrogenic stimulation in organs other than the kidneys, those which occurred in the pars intermedia of the pituitary gland are of particular interest. Several workers, (Cramer and Horning, 1936 ; Burrows, 1936 ; Deanesley, 1939 ; Gardner, 1947) have shown that prolonged oestrogenic stimulation induces chromophobe adenomas in most rodents. Vasquez-Lopez (1944) was the first to demonstrate that in the golden hamster the pituitary gland of both sexes reacts differently from that of other rodents to chronic oestrinisation. He succeeded in producing tumours of the pars intermedia which in several instances invaded the brain in the region of the tuber cinereum and in early stages resembled histologically the spontaneous basophilic invasion of the pars nervosa sometimes seen in the human hypophysis. The results of Vasquez-Lopez have been confirmed by Koneff, Simpson and Evans (1946) who implanted subcutaneously 10 mg. pellets of stilboestrol into hamsters of both sexes and obtained, after 3½ months' treatment, a hyperplasia of the pars intermedia which invaded progressively the pars posterior, the infundibulum and the floor of the third ventricle. They further found that the anterior lobe was invaded only after very prolonged treatment and that no histological changes occurred in the anterior lobe other than the infiltration by the pars intermedia cells. These results are in accord with our findings in hamsters which had been subjected to chronic oestrinisation for nearly 11½ months. The anterior lobe, except for invasion by cells of the pars intermedia, showed no diminution in the numbers of acidophiles and no increase in chromophobe cells.

The occurrence of hyperplastic foci in the adrenal cortex of several of the treated hamsters is very unusual. In laboratory animals, adrenal cortical tumours do not as a rule develop as the result of oestrogenic stimulation, Burrows and Horning (1952). In humans, tumours of the adrenal cortex arise more frequently in females than in males, and this clinical observation suggests that gonadal hormones may play some rôle in their aetiology. Therefore the occurrence of a nodular hyperplasia in some of the oestrogen-treated hamsters is of interest. There is only one other recorded instance of this. Gardner (1947) reported that in a strain of intact mice developing pituitary adenomas after prolonged oestrogen treatment, several had cortical hyperplasia in their adrenals. The lesions observed

by Gardner appear to be similar histologically to those in our hamsters. The occurrence of nodular hyperplasia of the adrenal cortex in oestrogen-treated rodents, though rare, is of particular interest as an indication that there may be some association between oestrinisation and the induction of adrenal tumours.

The effects of oestrogens on the reproductive system of rodents have been described by numerous workers. The histological changes in the prostate generally involve a rapid squamous metaplasia and keratinisation of the glandular epithelium, accompanied by an increase in the fibromuscular stroma. The hyperplasia induced in the alveolar epithelium of the ventral prostate of the oestrogen-treated hamster is a phenomenon normally never observed in other rodents. Moreover, in adjoining alveoli the prostatic epithelium undergoes the usual squamous changes in response to oestrogen. Why adjacent alveoli in the same prostatic lobe should respond differently is difficult to comprehend. The only other instance is that recorded by Burrows and Kennaway (1934) in a mouse treated with oestrone. The epithelial hyperplastic changes in these two cases are histologically identical with those occurring in man and the dog during enlargement of the prostate. These results are of interest as it is now contended that benign hypertrophy of the prostate in elderly men is the result of an excessive amount of naturally occurring oestrogen circulating in the blood stream, de Jongh (1935), Burrows (1935) and Teilum (1950).

## SUMMARY.

1. The histogenesis of stilboestrol-induced renal tumours in the intact male golden hamster has been described together with associated oestrogenic changes in other organs of the body.

2. The tumour foci arise from the epithelium of cortical tubules and grow by both expansion and peripheral infiltration. It is concluded that they are carcinomatous without structural demarcation between the earliest hyperplastic and

## EXPLANATION OF PLATES.

FIG. 1.—Macroscopic photograph of a stilboestrol-treated male hamster with bilateral and multifocal renal tumours, showing compact and cystic lesions. × 1¼.

FIG. 2.—Macroscopic photograph of a treated male hamster with large bilateral and multifocal kidney tumours. Note changes in epididymis. × 1¼.

FIG. 3.—Renal tumour showing trabecular and perivascular cell grouping. × 100.

FIG. 4.—Early tumour focus within the renal cortex. × 190.

FIG. 5.—Renal cortical tumour: an area with a compact, papillary structure. × 75.

FIG. 6.—Renal cortical carcinoma. Sarcoma-like area with a focus of calcification. × 80.

FIG. 7.—Periphery of renal cortical tumour showing inter-tubular infiltration. × 75.

FIG. 8.—Infiltration of peri-renal fat by superficially situated renal cortical carcinoma. × 80.

FIG. 9.—Renal tumour: an area of "clear" cells. × 190.

FIG. 10.—Renal tumour showing advanced cytoplasmic vacuolation and lipid deposits. × 190.

FIG. 11.—Pituitary tumour of the pars intermedia in a male hamster, showing cells in division. × 500.

FIG. 12.—*Ibid.* Tumour of pars intermedia cut through region of the hypophyseal cleft. Arrow indicates invasion of pars intermedia into the anterior lobe. × 77.

FIG. 13.—*Ibid.* Showing more detailed structure of the cells of the pars intermedia bordering the hypophyseal cleft. × 160.

FIG. 14.—Glandular hyperplasia in the prostate gland of a stilboestrol-treated hamster. × 75.

FIG. 15.—Squamous metaplasia of prostate epithelium in an adjoining alveolus. × 75.

FIG. 16.—Hyperplastic foci in the adrenal cortex of a stilboestrol-treated male hamster. × 80.

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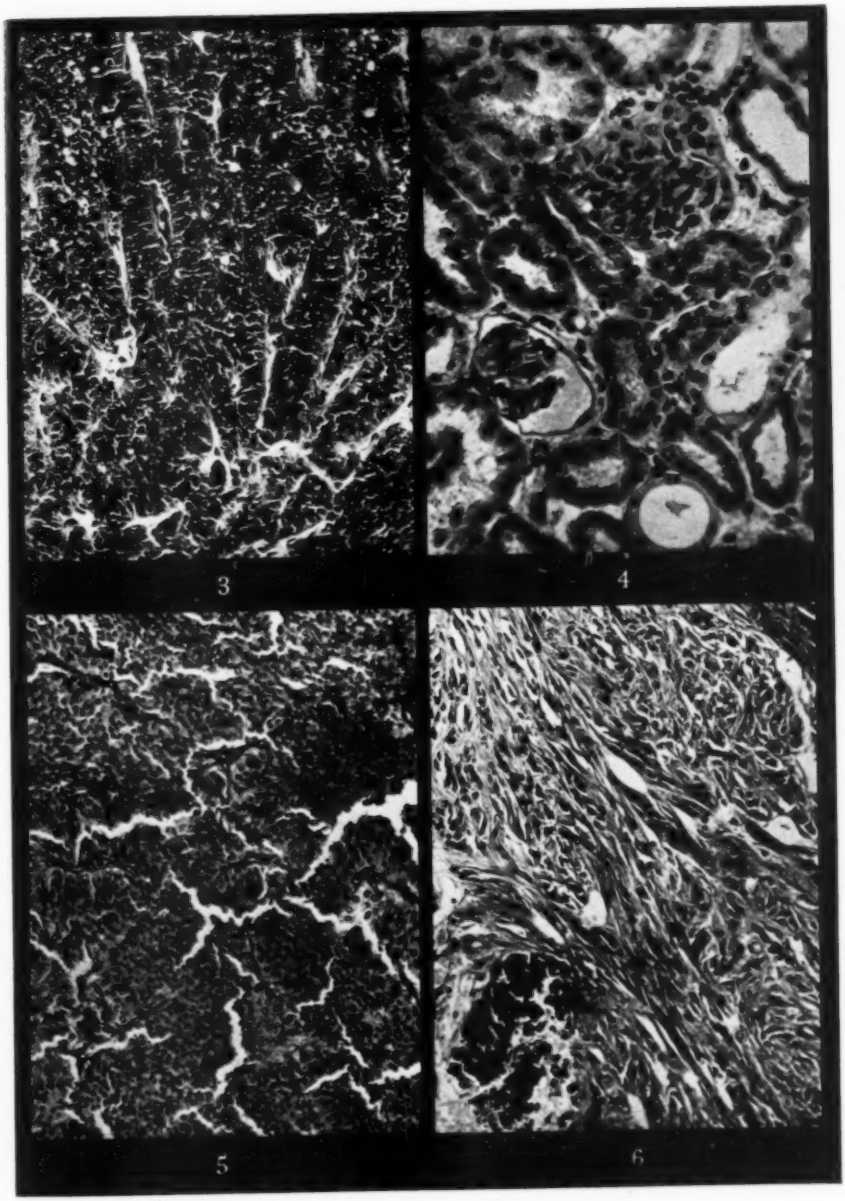

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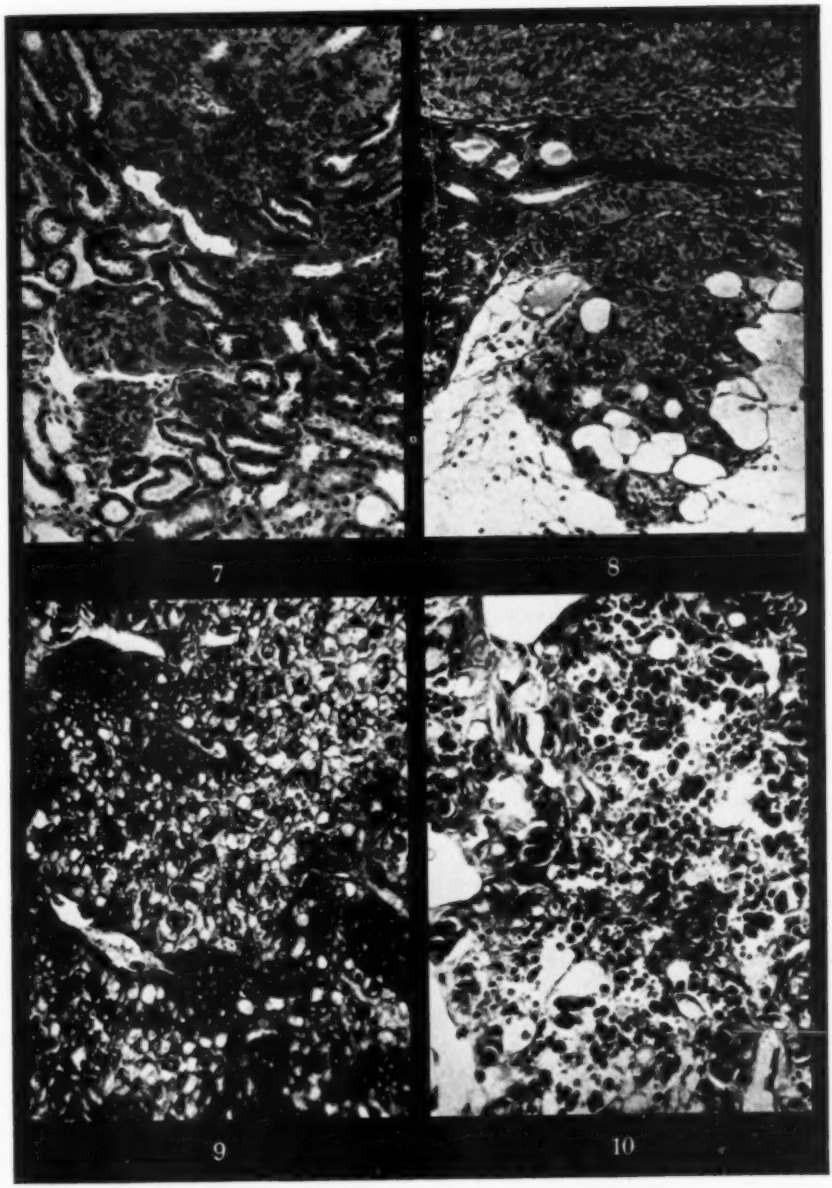

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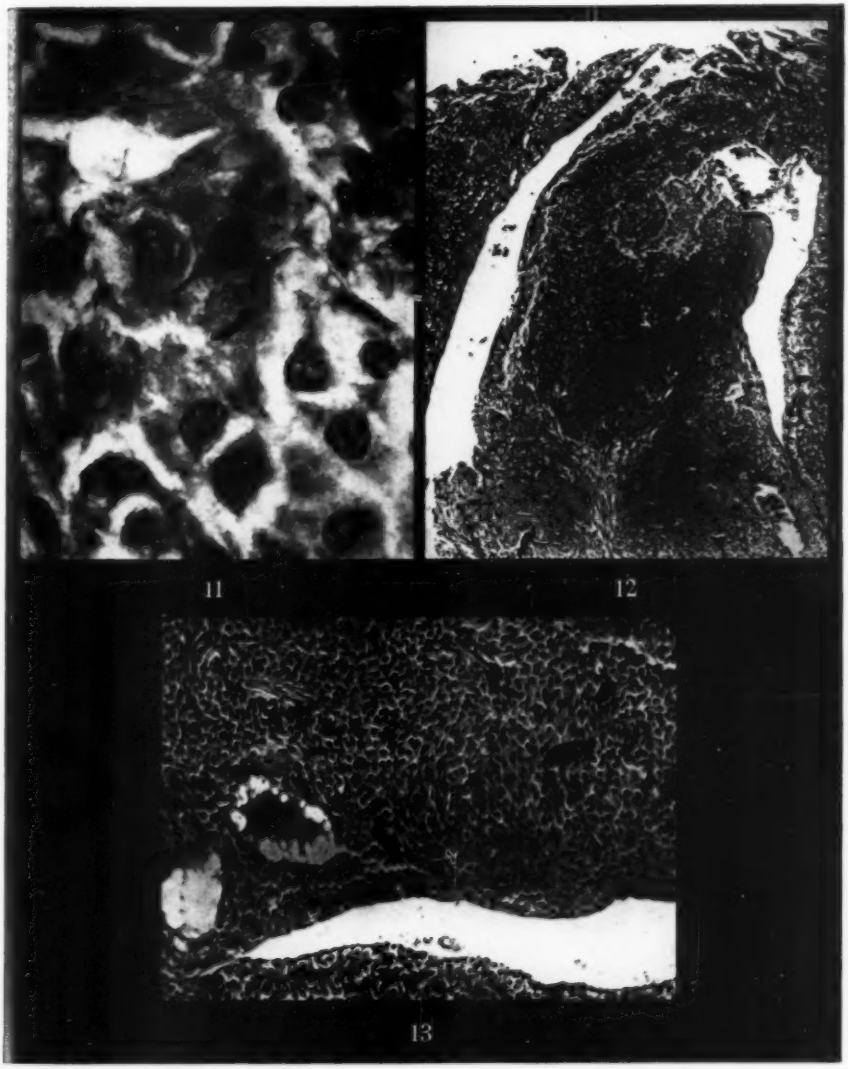

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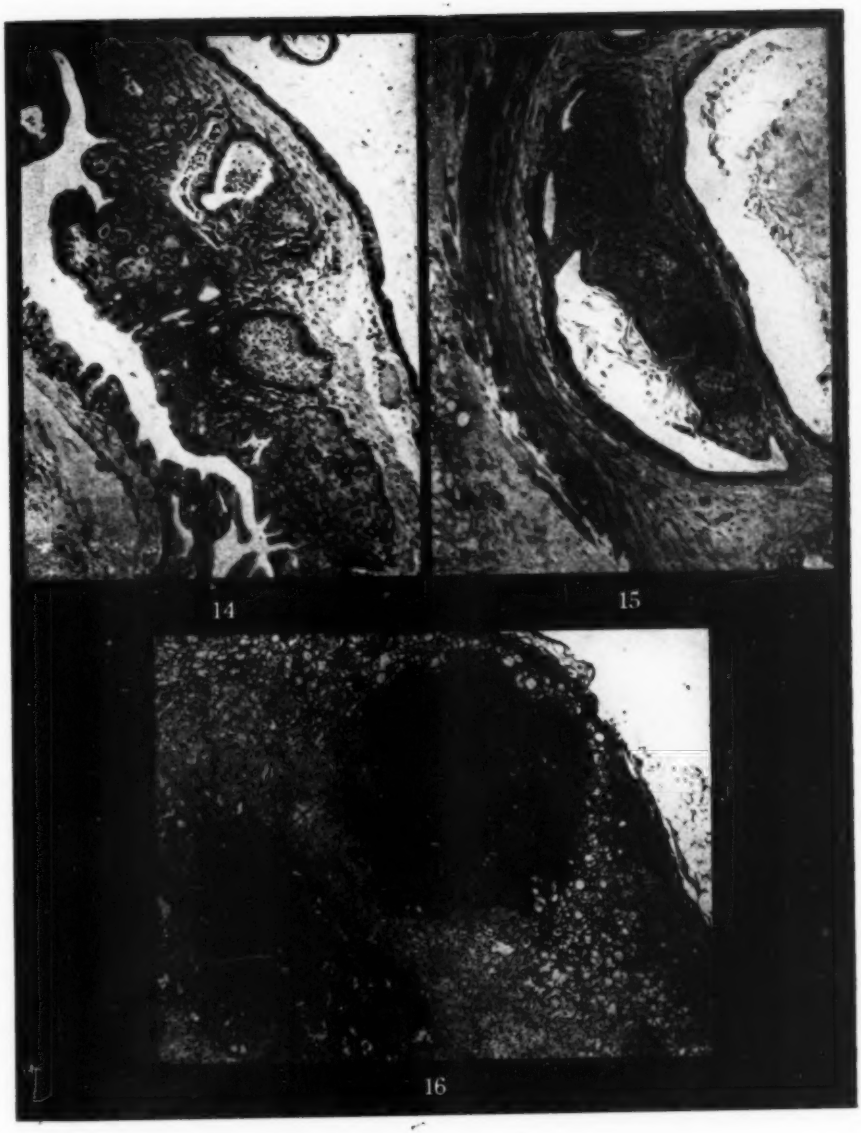

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final malignant states. The presence of intracellular, doubly refractile lipid is a constant and prominent feature in these tumours.

3. The most pronounced oestrogenic changes in organs other than the kidney occurred in the pituitary and adrenal glands. Twenty-eight out of 40 of the stilboestrol-treated male hamsters developed hypophyseal lesions of which 5 were tumours involving the pars intermedia. Hyperplastic foci of the adrenal cortex arose in several of the hamsters bearing pituitary tumours.

We are greatly indebted to Mr. D. Stephenson of the Burroughs Wellcome Foundation, for his kindness in supplying the 20 mg. diethylstilboestrol tablets.

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## REFERENCES.

Burrows, H.—(1936) *Amer. J. Cancer*, **28**, 741.—(1935) *J. Path. Bact.*, **41**, 218.
*Idem* and Horning, E. S.—(1952) 'Oestrogens and Neoplasia,' p. 67. Oxford (Blackwell Scientific Publications).
*Idem* and Kennaway, N. M.—(1934) *Amer. J. Cancer*, **70**, 48.
Cramer, W., and Horning, E. S.—(1936) *Lancet*, i, 247.
Deanesley, R.—(1939) *J. clin. Endocrin.*, **1**, 36.
de Jongh, S. E.—(1934) *Ned. Tijdschr. Geneesk.*, **78**, 489.—(1935) *Acta brev. neerl. Physiol.*, **5**, 28.
Gardner, W. U.—(1947) *Recent Progr. Hormone Res.*, **1**, 217.
Kirkman, H., and Bacon, R. L.—(1949) *Anat. Rec.*, **103**, 475.—(1950) *Cancer Res.*, **10**, 122.
Koneff, A. A., Simpson, M. E., and Evans, H. M.—(1946) *Anat. Rec.*, **94**, 169.
Korenchevsky, V., and Ross, M. A.—(1940) *Brit. Med. J.*, i, 645.
Lipschutz, A., Vargas, L., and Palma, J.—(1941) *J. Cancer Res.*, **1**, 575.
Matthews, V. S., Kirkman, H., and Bacon, R. L.—(1947) *Proc. Soc. exp. Biol., N.Y.*, **66**, 195.
Pfeiffer, C. A., Emmel, V. M., and Gardner, W. U.—(1940) *Yale J. Biol. Med.*, **12**, 493.
Teilum, G.—(1950) *Acta Endocrin.*, **4**, 43.
Van Wagenen, G.—(1935) *Anat. Rec.*, **63**, 387.
Vasquez-Lopez, E.—(1944) *J. Path. Bact.*, **56**, 1.
Wells, L. J.—(1936) *Anat. Rec.*, **64**, 475.
Zondek, B.—(1936) *Lancet*, i, 776.

# SENECIO ALKALOIDS: PRIMARY LIVER TUMOURS IN RATS AS A RESULT OF TREATMENT WITH (1) A MIXTURE OF ALKALOIDS FROM *S. JACOBAEA* LIN.; (2) RETRORSINE; (3) ISATIDINE.*

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COOK, Duffy and Schoental (1950) described the first experimental evidence in support of the view that consumption of Senecio alkaloids may be an aetiological factor in the high incidence of primary liver carcinoma among the African negroes. In a series of 11 rats, these workers observed primary liver tumours in the 3 males which survived longer than 8 months of intermittent feeding with a mixture of alkaloids from *S. jacobaea* Lin.

As there were so few survivors in this study, it was necessary to repeat these experiments with appropriate modifications of conditions and dosage, in order to ensure better survival of the animals.

*S. jacobaea* Lin. is, however, not a native plant in South Africa (Steyn, 1952). In view of the possible bearing of these results on the problem of primary liver carcinoma of the African negroes, it was important to test Senecio alkaloids from plants common in South Africa. The present communication describes conditions under which rats survived longer than 10 months of treatment with retrorsine and isatidine, alkaloids common in South Africa Senecio plants, and developed pathological changes ranging from nodular hyperplasia and fibrosis to neoplasia with metastases.

## MATERIAL AND METHODS.

Young, locally bred, albino Wistar rats which weighed 55–150 g. at the beginning of treatment were used. The animals, segregated by sex, were housed in groups in metal cages and were given a commercial cake diet and either water or the appropriate solutions of the alkaloids, *ad libitum*. In the early stages of the experiments with *S. jacobaea*, the rat cakes were supplied by the North Eastern Agricultural Co-operative Society Ltd., Aberdeen. When the animals were transferred to the Royal Beatson Memorial Hospital they received Shearer's Pig Weaner Nuts No. 1. The formulae of these two diets are as follows:—

*Preliminary report of a part of this investigation was communicated by R. S. to the 2nd International Congress of Biochemistry, Paris, 1952 (*Résumés des Communications*, p. 477); and at the Meeting of the American Association for Cancer Research, Chicago, 1953 (*Proc. Amer. Assoc. Cancer Res.*, 1953, **1**, 47; Corrigendum: (1953) *Cancer Res.*, **13**, 616.

| *Aberdeen Rat Cake Nuts.* | |
|---|---|
| Per cent. | |
| 19·2 | Thirds (wheat middlings). |
| 19·2 | Ground wheat. |
| 19·2 | Sussex ground oats. |
| 9·5 | Ground barley. |
| 9·5 | Ground maize. |
| 9·5 | Meat and bone meal (50 per cent protein). |
| 4·8 | Fish meal. |
| 7·0 | Milk powder (skimmed). |
| 1·3 | Dried yeast. |
| 0·4 | Cod liver oil. |
| 0·4 | Salt. |
| 100·0 | |

| *Shearer's Pig Weaner Nuts No. 1.* | |
|---|---|
| Per cent. | |
| 4·0 | Paisley meal. |
| 33·0 | Barley meal. |
| 7·5 | Ground oats. |
| 5·0 | White fish meal. |
| 10·0 | Indian meal. |
| 7·5 | Copra cake. |
| 22·0 | Bran. |
| 8·5 | Ground nut and Soya meal. |
| 2·5 | Minerals. |
| 100·0 | |

The animals were weighed at approximately weekly intervals till death. Controls were kept under the same conditions except for the treatment with the alkaloids. In a few animals biopsy specimens of the liver were taken under ether anaesthesia, for histological examination in order to follow the progression of the liver changes.

A new batch of ragwort supplied by a local herbalist was extracted, and the mixture of crystallised alkaloids at a concentration of 0·03—0·05 mg./ml. was used for the feeding experiments. Attempts at separation of individual components of this mixture led to the isolation of colourless rhombic crystals, mp. 232° C. (decomp.).* This melting point is higher than those of the pure alkaloids of *S. jacobaea* Lin. (Barger and Blackie, 1937). It is not unlikely that this batch of dried plants might have contained an admixture of another common weed, groundsel, *Senecio vulgaris*, the main alkaloid of which is senecionine, m.p. 232° C. (decomp.) (Barger and lBackie, 1936).

Pure crystalline retrorsine and isatidine, a generous gift from Professor F. L. Warren, Chemistry Department, University of Natal, Pietermaritzburg, were dissolved with the addition of equivalent amounts of dilute acid in the appropriate volume of water and stored at 0° C.–4° C. till used. The structure of these alkaloids, both of which occur in the same plants, and are widely represented among South African Senecio plants, has been established by Christie, Kropman, Leisegang and Warren (1949), and Leisegang and Warren (1950). Retrorsine is the *cis*-retronecic acid ester of retronecine, while isatidine is its N-oxide, *cis*-retronecic acid ester of retronecine-N-oxide.

Using concentrations of the alkaloids not exceeding 0·05 mg./ml., and rats older than 2 months, good survival of the animals was ensured. These solutions, unless mentioned otherwise, were given about 3 days weekly until the death of the animals. The rats survived from 10–24 months of such treatment; their weights did not differ significantly from those of the controls. Only shortly before death, the animals lost weight rapidly, developed yellowish, discoloured fur, and occasionally a distended abdomen due to ascites. The bladder was sometimes distended due to obstruction of the urethra by wax-like concretions, with accompanying overflow of urine.

* This compound may have been Jacoxine, mp. 228° C., recently identified as one of the five alkaloids isolated from Senecio Jacobaea, L., by R. B. Bradbury and C. C. J. Culvenor, *Chem. Ind.*, 1954, **33**, 1021.

1. *Alkaloids of* S. jacobaea, *Lin.*

Twenty-five rats (13 males and 12 females) were treated first with solutions containing 0·05 mg./ml. of the mixture of alkaloids during 1 week in the case of the males and during 2 weeks in the case of the females. Then the treatment was interrupted for 7 weeks during which time many of the young animals died. The treatment of the remaining 9 male and 1 female rats was resumed with solutions containing 0·03 mg./ml. given 3 days weekly till death, except in the case of 1 male which survived 7 months longer than the other animals, and during this time did not receive any further treatment.

2. *Retrorsine.*

Ten male and 4 female rats received solutions containing 0·03 mg./ ml. retrorsine 3 days weekly till death.

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EXPLANATION OF PLATES.

FIG. 1.—(606/52). Male rat treated with *S. jacobea* Lin. for 17 months. Nodular hyperplasia of liver. × 2. (after fixation).

FIG. 2.—(766/51). Male rat treated with *S. jacobaea* Lin. for 16 months. Two foci of hyperplasia separated by strands of fibrous tissue. Van Gieson. × 230.

FIG. 3.—(566/51). Male rat treated with *S. jacobaea* Lin. for 13½ months. Irregular area of early trabecular hepatoma. H. & E. × 70.

FIG. 4.—(1143/52). Male rat treated with retrorsine for 16 months. Granular appearance of under surface of liver and one nodule of hyperplasia, 5 mm. diameter. × 2.

FIG. 5.—(504/52). Male rat treated with retrorsine for 10 months. A nodule of hyperplasia with central haemorrhage. Early fibrosis and increased bile duct formation in the surrounding tissue. H. & E. × 30.

FIG. 6.—(959/53). Male rat treated with retrorsine for 14½ months. A round hepatoma 1 cm. diameter with areas of central haemorrhage. × 3½.

FIG. 7.—(959/53). Section showing a representative area of the tumour in Fig. 6. H. & E. × 190.

FIG. 8.—(789/52). Liver biopsy from male rat 959/52 after 12½ months' treatment with retrorsine, showing pale areas of degeneration surrounded by a zone of cellular hyperplasia. H. & E. × 27.

FIG. 9.—(350/53). Female rat treated with isatidine for 17 months. The tumour is composed of basophil cells in which mitoses are frequent. Proliferation of endothelial cells lining the hepatic vein is seen. Van Gieson. × 85.

Fig. 10.—(676/53). Female rat treated with isatidine for 19½ months. the tumour is composed of basophil cells surrounding an area of haemorrhage. H. & E. × 140.

FIG. 11.—(339/53). Male rat treated with isatidine for 14 months. Multiple tumours are present in all lobes of the liver. Metastases are seen in the pancreas and omentum. × 1½.

FIG. 12.—(339/53). Section of a hepatoma shown in Fig. 11. Anisocytosis and variation in nuclear size and staining are seen. H. & E. × 360.

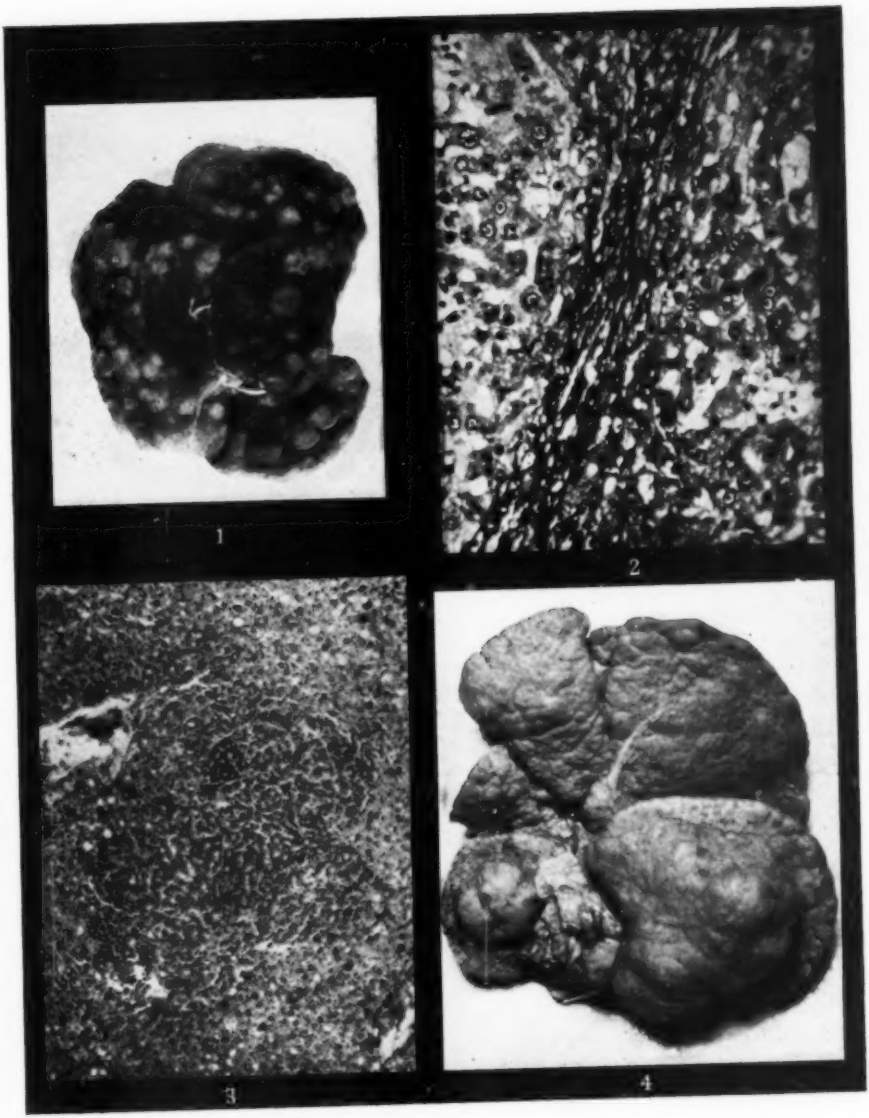
FIG. 13.—(339/53). Clumps of tumour cells are seen invading the wall of the bowel of the rat shown in Fig. 11. Post mortem change is present in the intestinal mucosa. H. & E. × 105.

FIG. 14.—(257/53). Male rat treated with isatidine and supplements of choline for 14 months. Trabecular hepatoma composed of basophil cells compressing the surrounding tissue. H. & E. × 95.

FIG. 15.—(257/53). A clump of tumour cells in a blood vessel from the same liver as shown in Fig. 14. H. & E. × 100.

FIG. 16.—(1065/53). Female rat treated with isatidine and choline supplements for 21½ months. Widespread tumour formation on the under surface of the liver. × 1½.

FIG. 17.—(985/53). Female rat painted with isatidine for 18½ months after initial intraperitoneal injection. Finger-like projections of liver cells covered by hypertrophied endothelial cells forming part of a hepatoma. H. & E. × 215.



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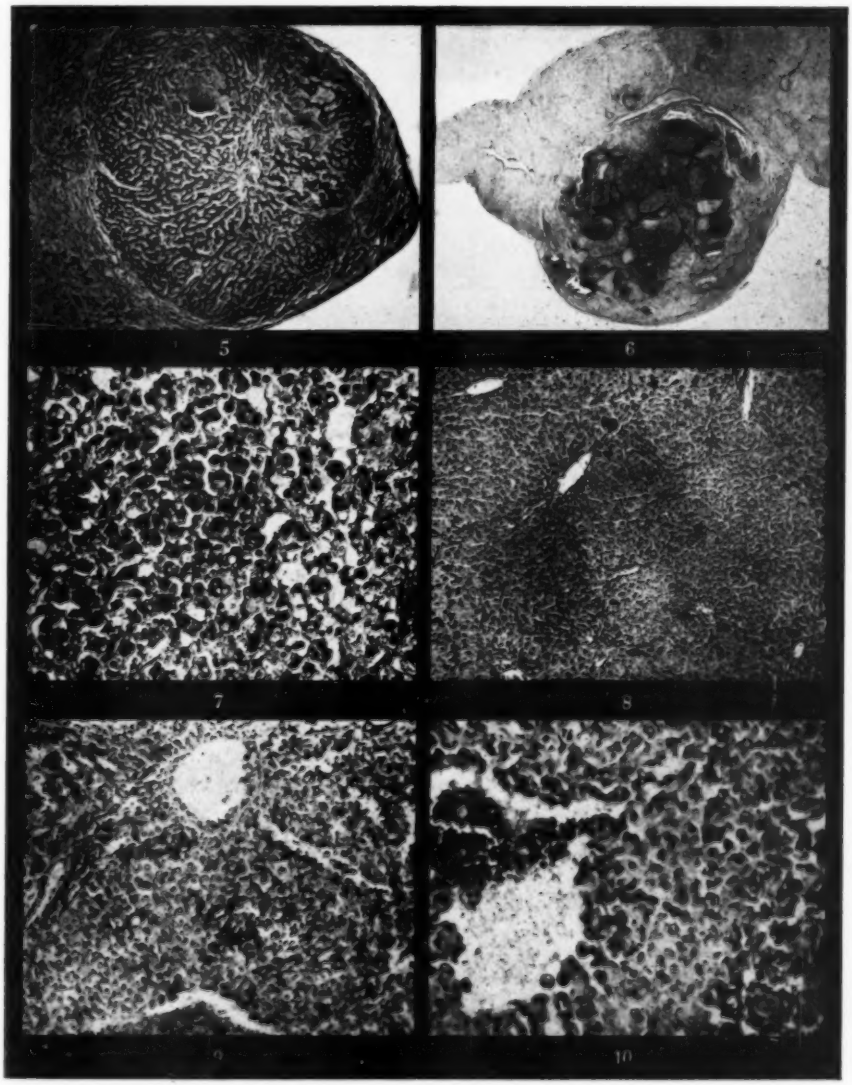

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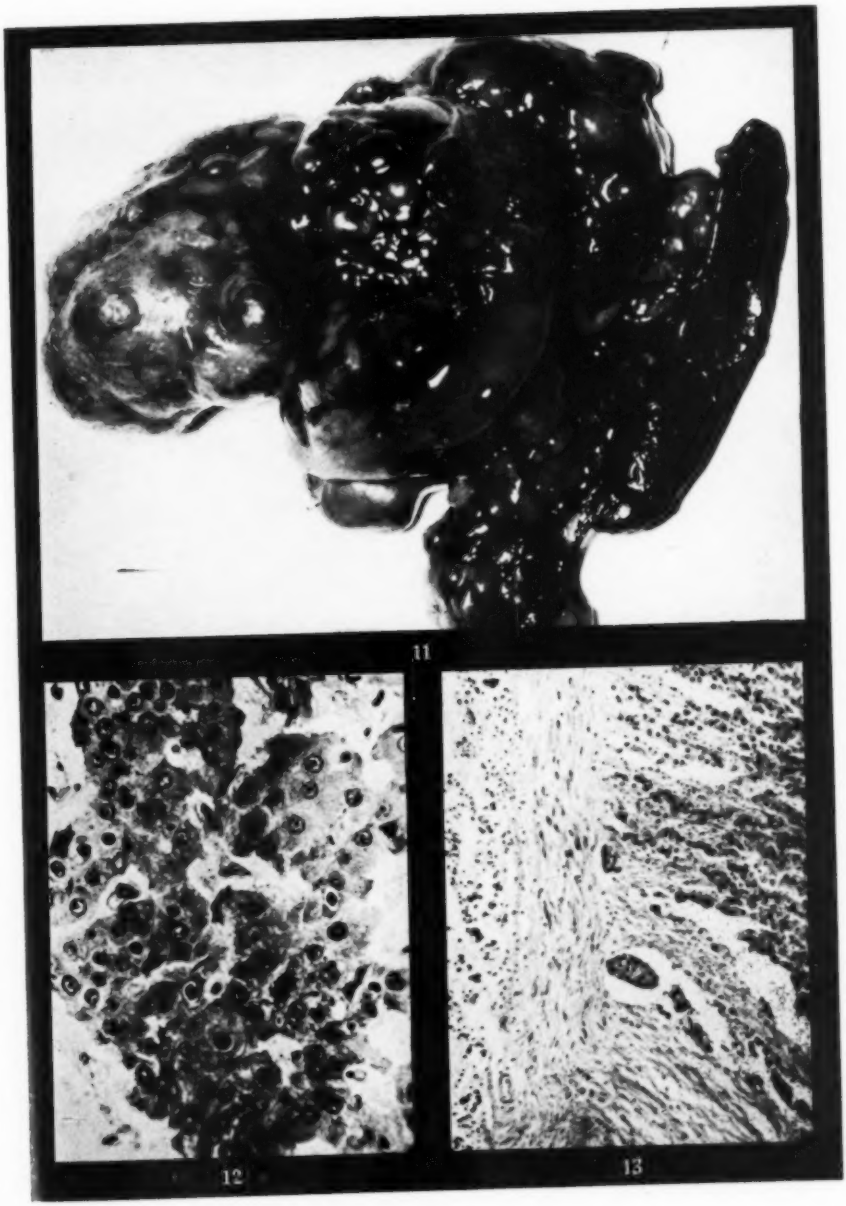

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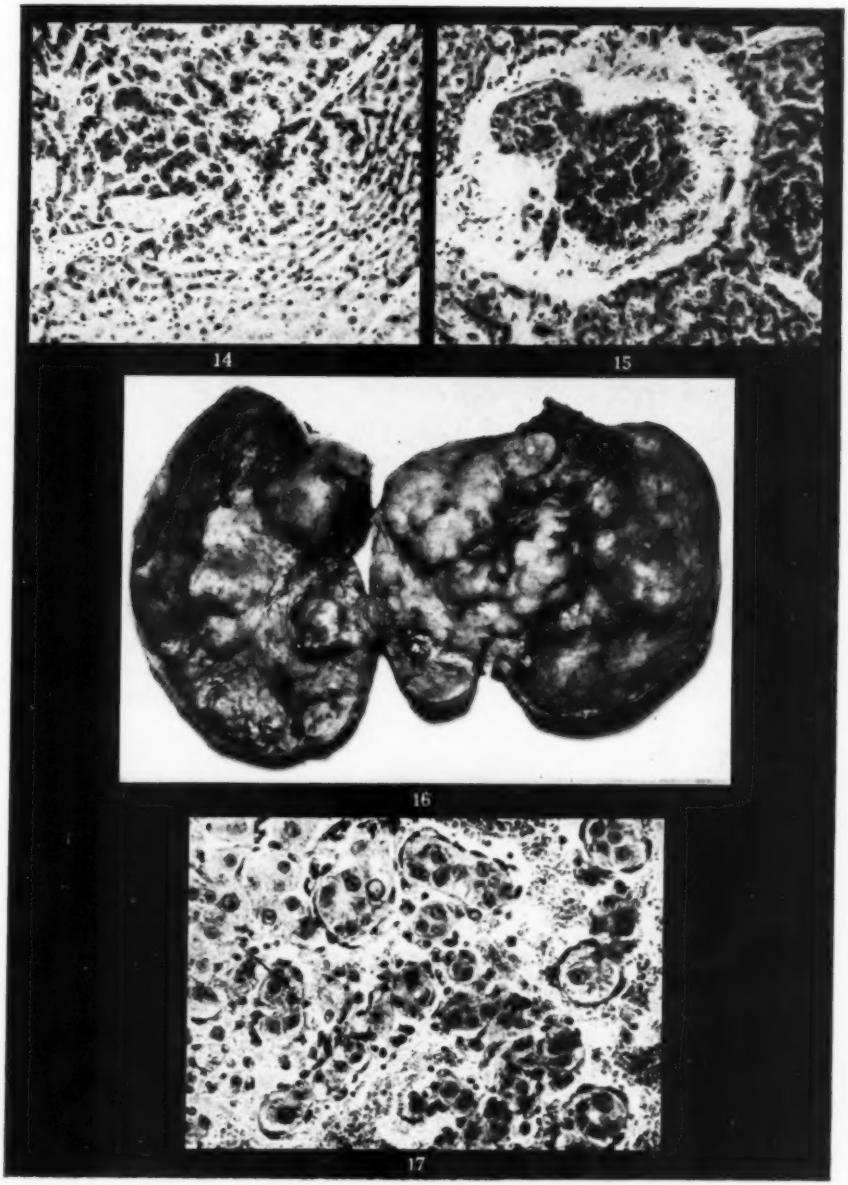

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3. *Isatidine* was given to 3 series of animals:

(*a*) Eight male and 14 female rats received solutions containing 0·05 mg./ml. in the early stages, followed later by solutions containing 0·03 mg./ml. 3 days weekly for about 20 months.

(*b*) Three male and 4 female rats received the same dosage of isatidine, but were given supplements of 0·5 per cent choline in drinking water during the remaining 4 days weekly till death.

(*c*) Two male and 3 female rats were given 1 intraperitoneal injection of 2 mg. isatidine in 0·2 ml. of tricaprylin followed by skin applications of a 0·5 per cent solution of isatidine in alcohol three times weekly for 15 months.

*Controls.*

Seven male and 7 female rats were kept as controls.

All the animals were examined post mortem. The livers and other organs were fixed in " formol corrosive," containing 1 part of commercial formalin and 9 parts saturated aqueous mercuric chloride, for microscopical examination. Sections were stained with haematoxylin and eosin; van Gieson stain was used for the demonstration of connective tissue; Gordon and Sweet's stain for reticulum and periodic acid—Schiff's stain for glycogen.

## RESULTS.

1. *Senecio Jacobaea.*

Nine male and 1 female rats survived from 11½ to 17 months from the start of treatment. On macroscopical examination all the male rats showed nodular hyperplasia of the liver, fairly uniformly distributed throughout the organ (Fig. 1); ascites was present in 4 cases, in one rat of 350 g. amounting to 125 ml. after 16½ months of treatment.

Histologically some of the nodules were seen to be composed of small regenerating cells without normal lobular arrangement. Others were formed of larger cells with central areas of haemorrhage. Areas of degenerating cells and large pools of haemorrhage were also present. There was increased bile duct formation and areas of cholangiofibrosis in the livers in all the male rats. Varying degrees of fibrosis round the portal tracts and interlobular veins were noted in all cases (Fig. 2). In 2 rats which died 13½ and 15½ months after the start of treatment, ascites was present and bladder concretions causing incontinence of urine. The liver showed cholangiofibrosis and nodular hyperplasia, and a few of these nodules had the appearance of early trabecular hepatoma (Fig. 3). The remaining rats in this series showed similar but less advanced changes in the liver which were difficult to classify. Although these were previously considered to be probably early hepatomata, they have now been regarded as an earlier stage in the progression from hyperplasia to neoplasia. The last rat of this series was treated for 17 months. Although it survived for 7 months longer without treatment, the liver still showed numerous small nodules and some pale areas projecting above the surface. On microscopical examination these were seen to be scattered foci of hyperplasia, areas of degenerating cells and of cystic bile duct formation. Much increase of bile duct production, cholangiofibrosis and fibrosis were present. In places there was variation in the size of the hepatic cells and in intensity of nuclear staining where one or two cells were surrounded by fibrous stroma.

The female rat in this series showed some degeneration and a few areas of compensatory hyperplasia in the liver.

2. *Retrorsine.*

Fourteen rats, 10 male and 4 female, were treated with retrorsine. On macroscopical examination of the 10 male rats, ascites was noted in 3 cases, 6 showed nodular hyperplasia and cirrhosis of the liver (Fig. 4), and in 3 others microscopical foci of hyperplasia were also found. Increased bile duct formation was noted in 9 cases, and fibrosis and cholangiofibrosis were found in 6 of these rats.

In 4 rats the nodules were found histologically to be hepatomata. In 2 of these which died 10 months after the start of treatment the livers were small, cirrhotic and nodular and ascites was present. On histological examination, areas of nodular hyperplasia (Fig. 5) and early hepatoma were seen. Increase in bile duct formation and of the endothelial cells lining the sinusoids were noted. In a third male rat which died 14½ months after treatment began, the whole liver was enlarged, congested and nodular and a haemorrhagic tumour 1 cm. diameter was seen on the under surface of the left lobe (Fig. 6). The spleen and kidneys were congested and ascites was present. On histological examination of the liver, areas of degeneration, regenation and haemorrhage were seen. The rounded mass was a hepatoma composed of a central area of haemorrhage surrounded by pale basophil cells in which mitoses were frequent (Fig. 7). Some small foci of similar appearance were seen in other parts of the liver. There was also an increase in bile duct formation. A liver biopsy carried out 2 months previously in this animal showed areas of degeneration and regeneration (Fig. 8).

The fourth male rat, which died 16 months after the start of treatment, showed nodular hyperplasia and hepatoma formation. Areas of increased bile duct formation and cholangiectasis were also present.

Of the 4 female rats, fatty degeneration of the liver was present in 2 which were killed after 17½ and 18 months. One killed after 23 months showed regenerative change in the liver and a papillary adenoma was present in the upper lobe of the left lung. The 4th rat died after 23 months without evidence of gross liver change.

3. *Isatidine.*

Thirty-four rats survived for more than 11 months of treatment with isatidine and 33 were examined histologically.

(*a*) Twenty-two of these rats, 8 male and 14 female, received 0·03 to 0·05 mg./ml. of isatidine in their drinking water; 7 showed no gross hepatic lesions and 15 showed nodularity of the liver, and of these, 10 (5 male and 5 female) contained multiple foci of tumour formation varying in size up to 10 mm. diameter. The other 5 showed merely areas of nodular hyperplasia where the cells were uniformly enlarged with clear cytoplasm and the appearances did not suggest neoplasia. Nevertheless we regard this hyperplasia as an early stage of a lesion which may progress; as the condition became more advanced larger nodules became apparent, each consisting of several hepatic lobules compressing the surrounding liver cells and these active foci stood out as pale areas. Areas of haemorrhage were some-

times found in their centres and the liver cords were separated by dilated sinusoids. In some livers, as Davidson (1935) previously observed, proliferation of the endothelial cells lining the sinusoids and hepatic veins was noted (Fig. 9), sometimes only one cell thick but in others forming tumour-like masses in which mitoses were frequent (Fig. 10). These changes were difficult to distinguish from hepatoma but the fine reticulin framework seen round the individual cells served to identify them. In only 1 rat did a metastasizing liver-cell tumour develop (Fig. 11 and 12); this was found 14 months after the start of treatment, and secondary growths were present in the pleura, omentum, pancreas, and invading the muscular coat of the bowel (Fig. 13).

Thus in this group of 15 animals the changes varied from simple hyperplasia through trabecular hepatoma to fully developed metastasizing carcinoma.

In 16 of the 22 rats of this group changes were found in the bile ducts varying from increase in number round the portal tracts to cholangiofibrosis, cholangiectasis and multilocular cysts. In 8 cases varying degrees of fibrosis were present.

(*b*) Seven rats, 3 males and 4 females, treated with isatidine and choline supplements survived from 14 to 21 months. All but 1 female showed nodular hyperplasia of the liver and 4 had prominent whitish nodules, from 3 to 10 mm. in diameter, interpreted histologically as trabecular hepatoma (Fig. 14 and 15). The last female of this series, killed 21½ months after the start of treatment, exhibited the most pronounced changes. The liver was enlarged, coarsely irregular, and on its under surface raised pale tumour-like masses were present (Fig. 16). On histological examination these areas were seen to be composed of trabecular hepatomata. Thus of the 6 rats which were killed or died earlier in this series, all showed changes in the liver, ranging from simple degeneration in 1, to regeneration with hyperplasia in 2, and finally to hepatoma in 3 cases. Areas of increased bile duct formation, cholangiectasis and fibrosis were seen in 5 rats. The administration of choline appeared to have had no effect in preventing the liver damage.

(*c*) Five rats, 2 male and 3 female, were painted with isatidine on the nape of the neck 3 times weekly after an initial intraperitoneal injection of isatidine, and survived from 11 to 18 months after the start of treatment. Some areas of degeneration and hyperplasia were noted in 2 males and 1 female. The last animal of this group, a female, was killed after 18½ months. The liver contained some whitish nodules; on histological examination these had the appearance of hepatoma in which finger-like projections of altered liver cells covered by prominent endothelial cells extended into a central haemorrhagic area (Fig. 17). This curious appearance was not uncommon in the trabecular hepatomata in the other series. No local skin changes were seen on the painted areas. Control rats which survived from 18½ to 25½ months showed no areas of hyperplasia in the liver.

## DISCUSSION.

Administration of Senecio alkaloids from *S. jacobaea* Lin. and the pure alkaloids retrorsine and isatidine from South African plants, by intermittent feeding or by painting on the skin, induced in rats profound liver damage.

If the dosage was adjusted to avoid acute toxic effects with high mortality within the first 3 months, it was possible to maintain the rats in apparently good health for periods of a year or more. Nevertheless such animals developed

extensive degenerative lesions accompanied or followed by regeneration, hyperplasia and in some cases by neoplasia of the liver parenchyma.

While the range of these pathological changes was similar to that already described in the case of rats fed on the mixed alkaloids of *S. jacobaea* Lin., the common ragwort of Great Britain (Cook, Duffy and Schoental, 1950) fibrosis was an additional feature in the present series of animals. This may have been due to the smaller dosage of alkaloid or to a change in diet from Aberdeen cake to Shearer's cake diet.

Cirrhosis though commonly associated with primary hepatoma in man and experimental animals, is not necessarily concomitant with hepatoma. As in the case of some azo dyes (Opie, 1944) purely hyperplastic and neoplastic epithelial growth might be encountered in some animals while in others cirrhosis, not accompanied by tumour growth, was found.

The failure of choline to protect rats from the action of isatidine indicates that different mechanisms may be involved in the induction of hepatoma in our rats as compared with those on a choline-deficient diet (Copeland and Salmon, 1946). As shown by Buckley, Buckley and Snipes (1951), supplements of choline did not prevent the development of liver tumours in rats due to feeding with butter yellow. On the contrary the incidence of hepatomata and metastases was increased.

No local changes followed skin painting with 0·5 per cent alcoholic solution of isatidine and the liver damage induced by this treatment was similar to, though less severe, than that induced by feeding with the same alkaloid.

As with most other hepatotrophic carcinogens, the female rats were less susceptible than the males to similar dosage of *S. jacobaea* and retrorsine. But in those treated with isatidine tumours were present not only in the males but also in the females. Clearly the mechanism of damage and repair in the liver is a highly complex matter and the visible results of a disturbed balance between many factors may be similar in several cases although the preceding sequence of events may have been very different. The mechanism of action of Senecio alkaloids is not yet known. It remains to be shown whether the hepatotoxic action is due to the parent alkaloid or to any of its metabolic products.

## SUMMARY.

Fifty-eight rats survived longer than 10 months of treatment with Senecio alkaloids from *S. jacobaea* Lin., retrorsine and isatidine. Forty-five of these cases showed changes in the liver ranging from hyperplasia to neoplasia. Metastases were found in 1 rat treated with isatidine.

Choline did not protect the liver from the action of isatidine.

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## REFERENCES.

BARGER, G., AND BLACKIE, J. J.—(1936) *J. Chem. Soc., p.* 743.—(1937) *Ibid.*, p. 584.
BUCKLEY, J. J., BUCKLEY, S. M., AND SNIPES, A. E.—(1951) *Johns Hopk. Hosp. Bull.*, **89**, 218.
CHRISTIE, S. M. H., KROPMAN, M., LEISEGANG, E. C., AND WARREN, F. L.—(1949) *J. Chem. Soc.*, p. 1700.
COOK, J. W., DUFFY, E., AND SCHOENTAL, R.—(1950) *Brit. J. Cancer*, **4**, 405.
COPELAND, D. H., AND SALMON, W. D.—(1946) *Amer. J. Path.*, **22**, 1059.
DAVIDSON, J.—(1935) *J. Path. Bact.*, **40**, 285.
LEISEGANG, E. C., AND WARREN, F. L.—(1950) *J. Chem. Soc.*, p. 702.
OPIE, E.—(1944) *J. exp. Med.*, **80**, 231.
STEYN, D. G.—(1952) *S. Afr. med. J.*, **26**, 139.

# THE EFFECT OF METHYLCHOLANTHRENE ON THE INCIDENCE OF LUNG TUMOURS IN TWO INBRED STRAINS OF MICE AND THEIR RECIPROCAL HYBRIDS.

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## METHODS.

RECIPROCAL crosses were made between the two inbred strains of mice, NBT and CBA, and the offspring were inbred for 12 generations. Half the members of each $F_1$ litter received one subcutaneous injection each of 1·0 mg. methylcholanthrene in 0·1 c.c. sesame oil at the age of 2 months, and their offspring were inbred for 11 more generations, the mice of $F_1$ to $F_{10}$ being injected. Details of the experiment have been described (Miller and Pybus, 1954). The injected $F_1$ mice and their descendants were designated the M/CBA/NBT and M/NBT/CBA strains (or MCN and MNC, for short) to distinguish them from the control CBA/NBT and NBT/CBA hybrids (CN and NC, for short), the maternal strain being named first. The last 2 generations, $F_{11}$ and $F_{12}$, of the MCN and MNC strains were not injected, neither were certain litters of each cross from $F_5$ onwards whose parents, at an early age, developed local tumours; these constituted the *uninjected* MCN and MNC groups.

Mice from the inbred parent strains were also injected, and subsequently bred from for one (uninjected) generation only. The injected mice formed the MCBA and MNBT groups, and their untreated offspring the MCBA $F_1$ and MNBT $F_1$ groups.

The main object of the experiment had been to obtain lines of hybrids resistant to the development of local tumours, in order to study the incidence of remote tumours, as had been done by Strong (1940, 1945), but no such resistant lines appeared.

In the following presentation of the results, data are given for the lung tumour incidence in the parent inbred strains and in the reciprocal hybrids. Within each group the tumour incidence in males and females are compared, as are also the ages at which tumours appeared in the two sexes, and the incidences and tumour ages of spontaneous tumours (in untreated mice) are examined and compared with the corresponding values for induced tumours (in mice injected with methylcholanthrene). Comparison is also made, for spontaneous and for induced tumours, between the 2 pure strains, and between the reciprocal hybrids.

The figures are based on the effective number of mice in each group (i.e., the number surviving at the time of discovery of the earliest lung tumour in that group). It is appropriate here to point out that, in common with other neoplasms of internal organs, the existence of a lung tumour is discovered only at death. As it is usually of slow growth and is only rarely the cause of death, the tumour may have

been present for a considerable time. Thus, in a comparison between average tumour ages in the various groups, if mice of one sex or of one strain are naturally shorter-lived, their average tumour age will be proportionately earlier. In the injected groups, where so many animals were killed young on account of local tumours, the "lung tumour age" (i.e., the age at which the tumours were discovered) probably corresponded more closely with the actual age of lung tumour development.

The lung tumours were of the normal type of adenoma or carcinoma so often described elsewhere; they varied in size from minute nodules to large growths occupying a whole lobe, and in the latter case could be the cause of death. The majority appeared to be of very slow growth.

Throughout this paper the word "tumour," without qualification, is to be understood to refer to lung tumour.

## RESULTS.

### 1. *Parent Pure Strains.*

#### A. *Controls.*

During the period of the experiment, mice from the 2 inbred strains were observed as controls; these came from Generations 25 to 36 of the CBA, and Generations 28 to 40 of the NBT. The tumour data are shown in Table I. In neither strain was the difference between the incidences in males and females significant.[1]* The difference between the strains, 6·0 per cent incidence in CBA compared with 2·1 per cent in NBT, was significant.[2]

Although the incidence was the same in both sexes, there was a significant difference in both strains between the average tumour ages in males and females,[3] on account of which it was impossible to take an average tumour age for each strain as a whole and the sexes were compared separately (Table I). The tumorous females of both strains died about 4 months later than the males.

Although the NBT mice were the more resistant, their average tumour age was over 10 months less than that of the CBA strain. The CBA mice (bred by Strong for long life (Strong, 1936)) lived to a much greater age than the NBT, and in both strains the females lived longer than the males; hence the tumours were found later in the females, and later in the CBA mice.

The incidences in both strains were low compared with those reported for Strain A (70–89 per cent) and Strain C (20–30 per cent) by Shimkin (1940*a*). The CBA mice were about as susceptible as the "moderately susceptible" C3H (7·0 per cent (Shimkin, 1940*a*)), while the incidence in the NBT mice was slightly higher than that of the "relatively resistant" $C_{57}B1$ strain (Shimkin, 1940*a*).

No record was kept of the number of spontaneous lung nodules per mouse in the pure strains, but the impression is that such tumours were usually solitary in both strains, but varied in size as already stated.

#### B. *Injected pure strains.*

The effect of methylcholanthrene was to raise the tumour incidence very greatly in both strains (Table I).

Out of 100 MCBA mice only 13 females and 17 males survived to the age of 12 months (earliest tumour age); 9 females and 8 males had tumours. The

* [1] and following numbers in this series refer to the notes on p. 484.

apparent sex-difference was not significant[4] and the total incidence of 56·7 per cent was almost 10 times that of the controls.

In the MNBT group, out of 131 mice only 49 females and 17 males survived to the earliest tumour age of 7 months, and of these 8 females and 2 males had tumours. Again the sex-difference was not significant[5] and the total incidence of 15·2 per cent was more than 7 times the control incidence.

The difference between the strain-incidences was significant,[6] MCBA mice being the more susceptible to this effect of the carcinogen. This is in agreement with the results for other inbred strains (Lynch, 1927 ; Andervont, 1937*a* ; Shimkin, 1940*a*) in which it was shown that susceptibility to induced tumours parallels that to spontaneous tumours.

The average tumour ages in males and females in the MCBA group did not differ significantly and both were several months earlier than in the controls (Table I).

TABLE I.—*The Incidence of Lung Tumours in the Two Inbred Strains of Mice, CBA and NBT, in the Methylcholanthrene-treated M/CBA and M/NBT, and in the Untreated Offspring of the Latter, M/CBA $F_1$ and M/NBT $F_1$.*

| Strain. | | Total effective number of mice. | Tumorous mice. Number. | Percentage. | | Average age at death (months). | | σ. | | Earliest tumour (months). | Latest tumour (months). |
|---|---|---|---|---|---|---|---|---|---|---|---|
| CBA | females | 243 | 19 | 7·8 | 5·95 | 26·3 | | 2·7 | | 14 | 31 |
| | males | 261 | 11 | 4·2 | | 22·5 | | 4·1 | | | |
| NBT | females | 287 | 5 | 1·7 | 2·1 | 16·0 | | 3·2 | | 5 | 20 |
| | males | 334 | 8 | 2·4 | | 11·9 | | 3·9 | | | |
| M/CBA | females | 13 | 9 | 69·2 | 56·7 | 16·7 | 17·0 | 3·5 | 3·8 | 12 | 24 |
| | males | 17 | 8 | 47·1 | | 17·4 | | 4·1 | | | |
| M/NBT | females | 49 | 8 | 16·3 | 15·2 | 14·8 | 13·7 | 2·6 | 3·3 | 7 | 18 |
| | males | 17 | 2 | 11·8 | | 9·5 | | 4·3 | | | |
| M/CBA | females | 167 | 8 | 4·7 | 4·9 | 27·6 | | 4·4 | | 17 | 34 |
| | males | 137 | 7 | 5·1 | | 21·3 | | 3·0 | | | |
| M/NBT $F_1$ | females | 73 | 8 | 11·0 | 7·0 | 16·3 | 15·8 | 3·1 | 3·4 | 9 | 19 |
| | males | 84 | 3 | 3·6 | | 14·3 | | 3·7 | | | |

There was no significant sex-difference in tumour age in the MNBT mice either, and although the average tumour age in each sex was less than that in the controls the difference was not significant.

In the absence of a sex-difference in tumour age, the ages for the two sexes could be averaged in each strain, and the differences of 3·3 months between the two injected groups was significant.[7]

It is impossible to be certain, under the conditions of this experiment, whether the carcinogen actually induced the tumours earlier or whether, as explained in the introduction, they were merely seen earlier. For an accurate comparison an equal number of control mice would have to be killed at the same time as the injected animals.

The development of induced multiple lung nodules is a well-known test of the tumour-susceptibility of a strain (Andervont, 1937*a* ; Lynch, 1940 ; Shimkin,

1940*a*; Heston, 1940). In the MCBA group, 2 of the 17 mice with induced tumours had solitary nodules, the remainder having 2 or more, an incidence of 11·8 per cent with solitary nodules. In the MNBT group, 6 of the 10 cases of induced tumours were solitary occurrences, an incidence of 60 per cent. The numbers in each class were small but the difference between the strains was significant[8] and confirmed the difference in susceptibility.

*c. The uninjected offspring ($F_1$) of each injected pure strain.*

There was no significant sex-difference in the tumour incidence in either group and the totals of 4·9 per cent in 304 MCBA $F_1$ mice and 7·0 per cent in 157 MNBT $F_1$ mice were in agreement. The MCBA $F_1$ tumour incidence did not differ significantly from that of 5·95 per cent in the CBA controls (Table I), but the MNBT $F_1$ tumour incidence showed a significant increase over the figure of 2·1 per cent in the NBT controls.[9]

This latter increase may have a genetical origin. Two pairs of injected MNBT mice (belonging to $F_{28}$ and $F_{29}$ of the strain), none of which showed a tumour, each had 3 tumorous "$F_1$" offspring, 3 out of 10 mice (equivalent to $F_{29}$ in the pure strain) from the first pair, and 3 out of 4 mice (equivalent to $F_{30}$) from the second. These two tumorous families both had close relations bearing tumours; in the first instance there were 2 spontaneous tumours in 9 $F_{27}$ mice, in the second there were 3 induced tumours amongst the 26 injected $F_{29}$ sibs of the injected parents. These two families may therefore have been more susceptible to lung tumours than were the remainder, for some reason such as a possible mutation in the line prior to treatment. The remaining 5 tumour mice of the 11 in MNBT $F_1$ were scattered singly and apparently at random amongst the families from the other 25 pairs of injected parents.

In the absence of a corresponding increase in tumour incidence in the MCBA $F_1$ group, there seems no reason to postulate the transmission of a carcinogenic effect from the injected parents.

An analysis of the tumour ages in the $F_1$ groups showed a sex-difference in MCBA but not in MNBT;[10] in the latter, the female tumour age followed the usual trend and was later than that of the males but not significantly so (owing to the small number of tumour males). The average tumour age in MCBA $F_1$ was many months later for both males and females compared with the corresponding MNBT $F_1$ animals. There was no significant difference in tumour age in either strain between the control and $F_1$ groups.

*Summary.*

The CBA strain was more susceptible both to spontaneous and to induced tumours than the NBT. There was no sex-difference in either strain in the incidence of either type of tumour. The average spontaneous tumour age was much later in the females than in the males in both strains and much later in the CBA than in the NBT; this difference could be due to the longer life of females than males and of CBA mice than NBT. The sex-difference in tumour age disappeared in the case of induced tumours but the strain-difference persisted, although much reduced compared with the controls. The tumour incidence and average tumour age in the generation of uninjected mice raised from the treated MCBA animals reverted to the control values, but in the MNBT $F_1$ group the incidence was raised

to the CBA level, probably owing to the inclusion of two unusually susceptible families. There were more mice with multiple lung nodules in the MCBA group than in the MNBT.

### 2. *Hybrid Strains ($F_1$ and $F_2$).*

The first two hybrid generations will be analysed separately. Analysis of the total spontaneous lung tumour incidence in all 12 generations of the reciprocal hybrids seemed to show that the NC mice were more susceptible than the CN (Miller and Pybus, 1952); this was not confirmed by an examination of the data for the first two generations.

A. *Spontaneous tumours.*

(1) *First hybrid generation.*—There was no significant difference between tumour incidences in the reciprocal hybrids (36·4 per cent in CN $F_1$ and 38·4 per cent in NC $F_1$) or in the two sexes in each group (Table II).

The $F_1$ mice lived longer than did those of later generations owing to hybrid vigour, and the average $F_1$ tumour ages were greater than the strain averages, but only in the CN males and the NC females were the differences in tumour age between $F_1$ and the strain large enough to be significant.[11] The earliest CN $F_1$ tumour was found at 18 months and the earliest NC $F_1$ tumour at 16 months.

Tumours were found significantly earlier in the males of each group.[12] Comparing the two groups, the average tumour ages for the females were in agreement, but the difference (2·7 months) between the males was significant.[13] This could be explained by the longer life of all CN $F_1$ males (23·2 months) compared with all NC $F_1$ males (19·7 months), tumours being found earlier in the latter.

(2) *Second hybrid generation.*—Again there was no sex-difference in tumour incidence, neither was the difference in incidence between the two groups significant (23·9 per cent in CN $F_2$, 18·7 per cent in NC $F_2$) (Table II).

There was no significant sex-difference in average tumour age in NC $F_2$, but, in CN $F_2$, tumours were found 3 months later in the males than in the females, a significant difference. Comparing the 2 groups, the average tumour ages for

TABLE II.—*Incidence of Spontaneous Lung Tumours in the First Two Generations of Two Reciprocal-hybrid Strains of Mice*

| Strain and generation. | Number of mice. | | Tumorous mice. | | | Average age at death (months). | σ. | Earliest tumour (months). | Latest tumour (months). |
|---|---|---|---|---|---|---|---|---|---|
| | Total. | Effective. | Number. | Percentage. | | | | | |
| CN $F_1$ females | 58 | 52 | 17 | 32·7 | 36·4 | 26·6 | 4·6 | 18 | 34 |
| CN $F_1$ males | 54 | 47 | 19 | 40·4 | | 23·9 | 2·6 | | |
| $F_2$ females | 145 | 127 | 26 | 20·5 | 23·9 | 24·8 | 2·7 | 16 | 30 |
| $F_2$ males | 116 | 99 | 28 | 28·3 | | 21·8 | 2·7 | | |
| NC $F_1$ females | 58 | 56 | 18 | 32·1 | 38·4 | 26·7 | 4·6 | 16 | 35 |
| NC $F_1$ males | 46 | 30 | 15 | 50·0 | | 21·2 | 2·7 | | |
| $F_2$ females | 109 | 105 | 24 | 22·9 | 18·7 | 25·0 | 3·3 | 12 | 30 |
| $F_2$ males | 129 | 114 | 17 | 14·9 | | 22·9 | 4·1 | | |

females were in agreement, as were also those for males. The earliest CN $F_2$ tumour was found at 16 months and the earliest NC $F_2$ tumour at 12 months.

Comparing $F_1$ and $F_2$, the NC $F_2$ incidence was significantly lower than the NC $F_1$,[14] but the difference between CN $F_1$ and $F_2$ was barely significant.[15] The mean tumour ages for the 2 generations were in agreement except for the CN males.[16] As a larger proportion of CN $F_2$ males died at an earlier age than did those of $F_1$, there was a greater opportunity for the discovery of early tumours in CN $F_2$.

The lower incidences in the $F_2$'s may be an indication of the segregation of factors concerned with tumour susceptibility, but there is another possible explanation which came to light when the data for induced tumours in the 2 generations were examined.

E. *Induced tumours.*

(1) *First hybrid generation.*—There was no significant difference between tumour incidences in the two sexes or in the reciprocal hybrids (46·5 per cent in MCN $F_1$ and 37·8 per cent in MNC $F_1$) (Table III). Neither was there a significant difference between the incidences of spontaneous and induced tumours in $F_1$. This latter result was unexpected and will be referred to again in the discussion; briefly, it raised the question of possible contamination of some $F_1$ mice with the carcinogen.

TABLE III.—*Incidence of Induced Lung Tumours in the First Two Generations of Two Reciprocal-hybrid Strains of Mice.*

| Strain and generation. | Number of mice. | | Tumorous mice. | | | Average at at death (months). | σ. | Earliest tumour (months). | Latest tumour (months). |
|---|---|---|---|---|---|---|---|---|---|
| | Total. | Effective. | Number. | Percentage. | | | | | |
| MCN $F_1$ females | 47 | 34 | 18 | 52·9 | 46·5 | 17·3 | 4·9 | 8 | 28 |
| MCN $F_1$ males | 50 | 37 | 15 | 40·5 | | 19·9 | 4·3 | | |
| $F_2$ females | 134 | 96 | 45 | 46·9 | 46·2 | 15·5 | 5·5 | 7 | 28 |
| $F_2$ males | 120 | 75 | 34 | 45·3 | | 13·9 | 4·5 | | |
| MNC $F_1$ females | 51 | 38 | 15 | 39·5 | 37·8 | 17·8 | 6·0 | 7 | 32 |
| MNC $F_1$ males | 54 | 36 | 13 | 36·1 | | 20·6 | 6·1 | | |
| $F_2$ females | 122 | 108 | 39 | 36·1 | 35·6 | 14·6 | 4·9 | 6 | 26 |
| $F_2$ males | 125 | 114 | 40 | 35·1 | | 15·7 | 5·1 | | |

The earliest MCN $F_1$ tumour was found at 8 months and the earliest MNC $F_1$ tumour at 7 months. Mean tumour ages in the injected mice were not significantly different in the two sexes or between the reciprocal hybrids, and were much lower than in the controls except in the MNC $F_1$ males; NC $F_1$ males died relatively young. Mean tumour ages were rather later than for the whole 10 generations of injected mice (*cf.* Table XVI), but significantly so only for the males (5·8 months later in MCN $F_1$, 6·9 months later in MNC $F_1$).[17]

(2) *Second hybrid generation.*—The induced tumour incidences in the reciprocal $F_2$'s were not significantly different (46·2 per cent in MCN $F_2$, 35·6 per cent in MNC $F_2$) (Table III), and there was no sex-difference.

There was no difference between mean tumour ages in the two sexes in either strain, neither did the tumour ages differ between the reciprocal hybrids.

The incidence of induced $F_2$ tumours was significantly higher than that of spontaneous $F_2$ tumours in the reciprocal crosses,[18] and induced tumours were found much earlier than spontaneous tumours in the $F_2$ mice.

Comparing $F_1$ and $F_2$, the incidences of induced tumours in the 2 generations were in agreement. Between $F_1$ and $F_2$ females the difference in induced-tumour age was not significant, but the difference between the males was significant,[19] owing to more of the $F_2$ males dying young and tumours being seen earlier.

c. *Multiple nodules.*

The post-mortem records were analysed for the occurrence of multiple nodules (Table IV). It was found that the majority of tumorous control mice in $F_1$ and $F_2$ of the reciprocal crosses had single lung nodules. The differences between the 2 strains and between the 2 generations in the same strain were not significant. In the injected $F_1$ and $F_2$ groups, the majority of the tumorous mice had multiple nodules (2 or more, the exact number over 2 not being stated in the records). The differences between the injected reciprocal hybrids, and between $F_1$ and $F_2$ in each injected group, were not significant, but the differences between the controls and the injected mice were significant.[20]

TABLE IV.—*Proportion of Tumour Mice Bearing Solitary Lung Nodules, in Methylcholanthrene-treated Animals and their Controls in Two Reciprocal-hybrid Crosses.*

| Strain. | Generation. | Number of tumour mice. | Mice with single lung nodules. | | Mice with two or more lung nodules. | |
|---|---|---|---|---|---|---|
| | | | Number. | Percentage. | Number. | Percentage. |
| CN | $F_1$ | 36 | 26 | 72·2 | 10 | 27·8 |
| | $F_2$ | 54 | 45 | 83·3 | 9 | 16·7 |
| | $F_1$–$F_{12}$ | 142 | 109 | 76·8 | 33 | 23·2 |
| MCN uninjected | $F_5$–$F_{12}$ | 40 | 31 | 77·5 | 9 | 22·5 |
| MCN injected | $F_1$ | 33 | 8 | 24·2 | 25 | 75·8 |
| | $F_2$ | 79 | 27 | 34·2 | 52 | 65·8 |
| | $F_1$–$F_{10}$ | 276 | 100 | 36·6 | 176 | 63·4 |
| NC | $F_1$ | 33 | 27 | 81·8 | 6 | 19·2 |
| | $F_2$ | 41 | 30 | 73·2 | 11 | 26·8 |
| | $F_1$–$F_{12}$ | 173 | 132 | 76·3 | 41 | 23·7 |
| MNC uninjected | $F_5$–$F_{12}$ | 169 | 155 | 91·7 | 14 | 8·3 |
| MNC injected | $F_1$ | 28 | 12 | 42·9 | 16 | 57·1 |
| | $F_2$ | 79 | 25 | 31·6 | 54 | 68·4 |
| | $F_1$–$F_{10}$ | 222 females | 76 | 34·2 | 146 | 65·8 |
| | | 165 males | 79 | 47·9 | 86 | 52·1 |

### 3. *Hybrid Strains (all generations).*

A. *Spontaneous tumours.*

The data for spontaneous lung tumour incidence and ages in all generations of the reciprocal hybrids are given in Tables V to XIII. The incidences are based on the number of mice living at the time of the earliest tumour in each group, so that the incidences in $F_1$ and $F_2$ (Tables V and IX), based on the numbers alive at 11 months, are less than those given in Table II in which the incidences (for

strict comparison of the individual generations) were calculated on the effective number of mice in each generation, *i.e.*, those alive at 18 and 16 months in the $F_1$'s and at 16 and 12 months in the $F_2$'s. Tables V and IX give the totals for all 12 generations and also for $F_2$ to $F_{12}$, *i.e.*, excluding the reciprocal $F_1$'s on account of the doubts mentioned above; the totals for $F_5$ to $F_{12}$ are given for comparison with the uninjected MCN and MNC groups.

(1) *CN and uninjected MCN hybrids (Tables V to VIII).*—There was no significant sex-difference in tumour incidence in either group, so the combined incidences for both sexes are given (Tables V and VI). The difference between the incidences in the 2 groups, 13·6 per cent in CN (or 11·3 per cent without $F_1$) and 5·6 per cent in uninjected MCN, was significant.[21] As the uninjected MCN animals came from Generations 5 to 12, the figures for these generations were compared. The totals of 28 tumours in 556 CN mice (5·0 per cent) and 40 tumours in 720 MCN uninjected mice (5·6 per cent) were in agreement. The high incidence in the whole CN strain was due to the high incidence in the early generations, from which there was a marked fall to a moderately low incidence with considerable fluctuation about

TABLE V.—*Incidence of Spontaneous Lung Tumours in each Generation of CN Hybrids.*

| Generation. | Number of mice. Total in experiment. | Number of mice. Surviving to tumour age. | Tumorous mice. Number. | Tumorous mice. Percentage. |
|---|---|---|---|---|
| $F_1$ | 112 | 107 | 36 | 33·6 |
| $F_2$ | 261 | 248 | 54 | 21·8 |
| $F_3$ | 76 | 66 | 16 | 24·2 |
| $F_4$ | 83 | 65 | 8 | 12·3 |
| $F_5$ | 71 | 44 | 4 | 9·1 |
| $F_6$ | 53 | 33 | 2 | 6·1 |
| $F_7$ | 63 | 41 | 4 | 9·8 |
| $F_8$ | 96 | 68 | 2 | 2·9 |
| $F_9$ | 100 | 48 | 7 | 14·6 |
| $F_{10}$ | 113 | 63 | 2 | 3·2 |
| $F_{11}$ | 130 | 51 | 1 | 2·0 |
| $F_{12}$ | 275 | 208 | 6 | 2·9 |
| $F_1$–$F_{12}$ | 1433 | 1042 | 142 | 13·6 |
| $F_2$–$F_{12}$ | 1321 | 935 | 106 | 11·3 |
| $F_5$–$F_{12}$ | 901 | 556 | 28 | 5·0 |

TABLE VI.—*Incidence of Spontaneous Lung Tumours in each Generation of Uninjected MCN Hybrids.*

| Generation. | Number of mice. Total in experiment. | Number of mice. Surviving to tumour age. | Tumorous mice. Number. | Tumorous mice. Percentage. |
|---|---|---|---|---|
| $F_5$ | 92 | 86 | 10 | 11·6 |
| $F_6$ | 12 | 5 | 1 | 20·0 |
| $F_7$ | 44 | 40 | 2 | 5·0 |
| $F_8$ | 81 | 69 | 4 | 5·8 |
| $F_9$ | 107 | 75 | 3 | 4·0 |
| $F_{10}$ | 109 | 78 | 7 | 9·0 |
| $F_{11}$ | 222 | 149 | 9 | 6·0 |
| $F_{12}$ | 275 | 218 | 4 | 1·8 |
| $F_5$–$F_{12}$ | 942 | 720 | 40 | 5·6 |

that figure in the last 8 generations of both groups, doubtless due to a segregation of factors for susceptibility and a chance breeding of the less susceptible lines.

Tumours were found much earlier in the uninjected MCN mice than in the CN group as a whole (Table VII). Taking the last 8 generations of the CN hybrids, the difference between the males was reversed owing to the shorter life of CN males in the later generations. Table VIII gives the mean ages at death of all the CN and uninjected MCN mice; the males died earlier than the females, the difference within each group being significant[22], [23]. The differences between the 2 groups were also significant.[24] The earlier tumour age in the uninjected MCN group was therefore due to the mice of that group dying earlier and the tumours being found sooner.

TABLE VII.—*Ages at Death of Tumour Mice in Control CN and Uninjected MCN Hybrids.*

| | | Tumour females. | | | Tumour males. | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Strain. | Generation. | Number. | Average age at death (months). | σ. | Number. | Average age at death (months). | σ. | Earliest tumour (months). | Latest tumour (months). |
| CN | $F_1$–$F_{12}$ | 76 | 24·7 | 4·5 | 66 | 22·0 | 3·8 | 11 | 34 |
| | $F_5$–$F_{12}$ | 21 | 23·3 | 5·1 | 7 | 15·3 | 2·2 | 12 | 32 |
| MCN uninjected | $F_5$–$F_{12}$ | 25 | 16·3 | 3·2 | 15 | 17·4 | 3·9 | 11 | 24 |

TABLE VIII.—*Ages at Death of Control CN and Uninjected MCN Mice.*

| | | Females. | | | Males. | | |
|---|---|---|---|---|---|---|---|
| Strain. | Generation. | Total in experiment. | Average age at death (months). | σ. | Total in experiment. | Average age at death (months). | σ. |
| CN | $F_1$–$F_{12}$ | 706 | 19·4 | 7·2 | 727 | 13·5 | 6·6 |
| MCN uninjected | $F_5$–$F_{12}$ | 438 | 13·7 | 4·4 | 504 | 12·4 | 4·1 |

(2) *NC and uninjected MNC hybrids. (Tables IX to XIII).*—The tumour incidences for the whole 12 generations of NC hybrids (Table IX) were 21·8 per cent in females and 12·9 per cent in males, or 20·6 per cent and 10·4 per cent without $F_1$, both significant sex-differences, [25] and this was the only group in the whole experiment to show a sex-difference in the incidence of spontaneous tumours.

In the uninjected MNC group the tumour incidences in females (9·9 per cent) and males (9·0 per cent) were in agreement and Table X gives the incidences for the two sexes combined. For the corresponding generations, $F_5$ to $F_{12}$, the NC incidences were 17·4 per cent (females) and 8·4 per cent (males). Comparing the 2 groups, the tumour incidences in the females differed significantly[26] but those in the males were in agreement.

For all the NC mice in the experiment, the mean age at death of the males was much less than that of the females (Table XI), the difference of 5·9 months being significant;[27] 186 males but only 51 females died before the age of 11 months. Table XII shows the result of a simple calculation based on the numbers of mice alive at 11 months and over, whereby it was found (Column 6) that, if the males

TABLE IX.—*Incidence of Spontaneous Lung Tumours in each Generation of NC Hybrids.*

| Generation. | Number of mice. | | Tumorous mice. | | Females only. | | | Males only. | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Total. | Effective. | Number | Percentage. | Effective number of mice. | Number with tumours. | Percentage tumour incidence. | Effective number of mice. | Number with tumours. | Percentage tumour incidence. |
| $F_1$ | 104 | 97 | 33 | 34·0 | 57 | 18 | 31·6 | 40 | 15 | 37·5 |
| $F_2$ | 238 | 220 | 41 | 18·1 | 105 | 24 | 22·9 | 115 | 17 | 14·5 |
| $F_3$ | 108 | 84 | 13 | 15·5 | 54 | 11 | 20·4 | 30 | 2 | 6·7 |
| $F_4$ | 119 | 101 | 23 | 22·8 | 58 | 18 | 31·8 | 43 | 5 | 11·6 |
| $F_5$ | 96 | 82 | 9 | 11·0 | 38 | 7 | 18·4 | 44 | 2 | 4·5 |
| $F_6$ | 83 | 68 | 10 | 14·7 | 43 | 9 | 20·9 | 25 | 1 | 4·0 |
| $F_7$ | 72 | 58 | 8 | 13·8 | 22 | 4 | 18·2 | 36 | 4 | 11·1 |
| $F_8$ | 118 | 84 | 14 | 16·7 | 47 | 10 | 21·3 | 37 | 4 | 10·8 |
| $F_9$ | 83 | 61 | 8 | 13·1 | 33 | 4 | 12·1 | 28 | 4 | 14·3 |
| $F_{10}$ | 83 | 47 | 1 | 2·1 | 27 | 0 | 0·0 | 20 | 1 | 5·0 |
| $F_{11}$ | 52 | 24 | 9 | 37·5 | 21 | 8 | 38·1 | 3 | 1 | 33·3 |
| $F_{12}$ | 56 | 49 | 4 | 8·2 | 27 | 3 | 11·1 | 22 | 1 | 4·5 |
| $F_1$–$F_{12}$ | 1212 | 975 | 173 | 17·7 | 532 | 116 | 21·8 | 443 | 57 | 12·9 |
| $F_2$–$F_{12}$ | 1108 | 878 | 140 | 15·9 | 475 | 98 | 20·6 | 403 | 42 | 10·4 |
| $F_3$–$F_{12}$ | 643 | 473 | 63 | 13·3 | 258 | 45 | 17·4 | 215 | 18 | 8·4 |

TABLE X.—*Incidence of Spontaneous Lung Tumours in each Generation of Uninjected MNC Hybrids.*

| Generation. | Number of mice. | | Tumorous mice. | |
|---|---|---|---|---|
| | Total in experiment. | Surviving to tumour age. | Number. | Percentage. |
| $F_5$ | 84 | 56 | 13 | 23·2 |
| $F_6$ | 217 | 200 | 22 | 11·0 |
| $F_7$ | 154 | 148 | 9 | 6·1 |
| $F_8$ | 88 | 85 | 9 | 10·6 |
| $F_9$ | 81 | 76 | 5 | 6·6 |
| $F_{10}$ | 163 | 150 | 15 | 10·0 |
| $F_{11}$ | 364 | 327 | 21 | 6·7 |
| $F_{12}$ | 781 | 755 | 75 | 9·9 |
| $F_5$–$F_{12}$ | 1932 | 1797 | 169 | 9·4 |

had lived as long as the females, there would have been 113 tumorous males (a 25·5 per cent incidence), which agrees well with the incidence of 21·8 per cent in females. It is proposed to take the tumour incidence in the females as the true incidence for this group.

TABLE XI.—*Ages at Death of Control NC and Uninjected MNC Mice.*

| Strain. | Generation. | Females. | | | Males. | | |
|---|---|---|---|---|---|---|---|
| | | Total in experiment. | Average age at death (months). | σ. | Total in experiment. | Average age at death (months). | σ. |
| NC | $F_1$–$F_{12}$ | 583 | 20·8 | 6·3 | 629 | 14·9 | 6·5 |
| | $F_5$–$F_{12}$ | 293 | 17·9 | 5·5 | 350 | 12·5 | 5·0 |
| MNC uninjected | $F_5$–$F_{12}$ | 865 | 19·6 | 5·5 | 1067 | 17·5 | 4·8 |

TABLE XII.—*Calculation of Expected Number of Spontaneous Lung Tumours in NC Male Mice, if the Males had Lived as Long as the Females.*

| Age at death (months). (1) | Percentage of mice alive at the beginning of each month. Females. (2) | Males. (3) | Females. / Males. (4) | Number of tumour males. (5) | Columns 4 × 5. (6) | Number of tumour females. (7) |
|---|---|---|---|---|---|---|
| 11 | 100·0 | 100·0 | 1·00 | 2 | 2·00 | 0 |
| 12 | 97·4 | 91·2 | 1·07 | 2 | 2·14 | 0 |
| 13 | 95·3 | 86·0 | 1·11 | 2 | 2·22 | 2 |
| 14 | 92·8 | 77·9 | 1·19 | 1 | 1·19 | 1 |
| 15 | 89·8 | 70·9 | 1·27 | 1 | 1·27 | 1 |
| 16 | 85·9 | 66·6 | 1·29 | 3 | 3·87 | 2 |
| 17 | 81·6 | 61·0 | 1·34 | 2 | 2·68 | 2 |
| 18 | 77·8 | 52·8 | 1·47 | 4 | 5·88 | 5 |
| 19 | 72·6 | 44·0 | 1·65 | 7 | 11·55 | 4 |
| 20 | 68·2 | 35·0 | 1·95 | 4 | 7·80 | 7 |
| 21 | 62·6 | 28·7 | 2·18 | 7 | 15·26 | 12 |
| 22 | 53·9 | 24·4 | 2·21 | 4 | 8·84 | 12 |
| 23 | 45·7 | 19·0 | 2·41 | 5 | 12·05 | 11 |
| 24 | 37·8 | 15·8 | 2·39 | 3 | 7·17 | 12 |
| 25 | 30·3 | 13·5 | 2·24 | 6 | 13·44 | 8 |
| 26 | 22·7 | 8·6 | 2·64 | 1 | 2·64 | 9 |
| 27 | 17·7 | 5·0 | 3·54 | 1 | 3·54 | 6 |
| 28 | 13·7 | 2·9 | 4·73 | 2 | 9·46 | 6 |
| 29 | 7·7 | 2·0 | 3·85 | 0 | 0·00 | 6 |
| 30 | 5·3 | 1·6 | 3·31 | 0 | 0·00 | 6 |
| 31 | 3·0 | 1·1 | 2·73 | 0 | 0·00 | 2 |
| 32 | 1·9 | 0·2 | 9·50 | 0 | 0·00 | 0 |
| 33 | 1·3 | 0·0 | ∞ | 0 | 0·00 | 1 |
| 34 | 1·1 | 0·0 | ∞ | 0 | 0·00 | 0 |
| 35 | 0·8 | 0·0 | ∞ | 0 | 0·00 | 1 |
| 36 | 0·2 | 0·0 | ∞ | 0 | 0·00 | 0 |
| Total number of tumours | | | | 57 | 113·00 | 116 |

Table XI shows that the mean ages at death of all the MNC uninjected mice were higher than the corresponding mean ages for NC ($F_5$ to $F_{12}$), the differences being significant for each sex.[28]

Mean tumour ages in the uninjected MNC mice showed no sex-difference (Table XIII). Tumours were found later in the uninjected MNC mice than in the corresponding generations of NC mice, the differences between the 2 groups being significant for each sex.[29]

TABLE XIII.—*Ages at Death of Tumour Mice in Control NC and Uninjected MNC Hybrids.*

| Strain. | Generation. | Tumour females. Number. | Average age at death (months). | σ. | Tumour males. Number. | Average age at death (months). | σ. | Earliest tumour (months). | Latest tumour (months). |
|---|---|---|---|---|---|---|---|---|---|
| NC | $F_1$–$F_{12}$ | 116 | 23·6 | 3·2 | 57 | 20·2 | 3·9 | 11 | 35 |
| | $F_5$–$F_{12}$ | 45 | 20·6 | 3·5 | 18 | 16·4 | 3·3 | 11 | 27 |
| MNC uninjected | $F_5$–$F_{12}$ | 80 | 22·2 | 4·0 | 89 | 21·1 | 3·9 | 9 | 29 |

Since the uninjected MNC mice lived longer than the corresponding generations of NC mice, (as much as 5 months longer in the case of the males), the tumour incidence should have been higher in the former than in the latter if the two groups had been equally susceptible. The very much lower incidence in the uninjected MNC mice denotes that the latter were much less susceptible.

Examination of the heredity charts showed that certain lines of NC hybrids appeared to have fewer cases of lung tumours, sometimes probably owing to earlier deaths of the mice from other causes, but one line in particular had a definitely higher incidence persisting through all 12 generations. It was explained in a previous paper (Miller and Pybus, 1954) that, although the original inbred parents of the hybrids were mostly very closely related within their own strains, there was a possibility of genetic difference between those coming from two lines of the CBA strain. The MNC uninjected mice were, with the exception of 41 $F_5$ animals, all the product of one type of cross (C × P) as previously explained (Miller and Pybus, 1954). An analysis was made of the offspring of the same cross forming part of the NC group, and for strict comparison Generations 5 to 12 were taken. There were 4 lung tumours in 32 females and 5 in 41 males and, as these incidences were in agreement, the total incidence of 9 in 73 mice (12·3) per cent was used; this was not significantly different from the total incidence in the uninjected MNC group of 169 in 1797 mice (9·4 per cent). This confirms the opinion that the lower incidence in the uninjected MNC group (compared with the total NC group) was due to a genetical difference from the majority of the NC hybrids.

Comparing the reciprocal hybrids CN and NC as a whole, the NC mice appeared to be more susceptible to spontaneous lung tumours, the difference between the incidences in the females being significant,[30] but any real difference between the males was masked by the early death of the majority of NC males. The apparent difference between the two groups seems to be due to a segregation of more resistant lines in the later CN generations and of more susceptible lines in the later NC generations.

The total incidences in the uninjected mice of the MCN and MNC groups also differed significantly.[31] As previously mentioned (Miller and Pybus, 1954), although the pure strain parents of the hybrids came from several different inbred lines, the bulk of the MCN and MNC mice were the offspring of reciprocal crosses between NBT Line C and CBA Line P. Since all the MCN and MNC mice of the first 4 generations were injected, there were no uninjected animals of these 2 groups available for comparison until $F_5$, by which time lines of different susceptibilities could have segregated. There was considerable fluctuations in incidence from generation to generation (Tables VI and X) and the actual numbers of tumours in each generation, especially in the uninjected MCN group, were often too small for individual analysis. The uninjected MCN and MNC mice were better controls, genetically, for the later generations of the injected groups than were the CN and NC hybrids.

B. *Induced tumours.*

The data for induced lung tumour incidences in all generations of the reciprocal hybrids are given in Tables XIV and XV. For the sake of comparison between the whole groups, the figures are based on the numbers of mice alive at the time of the earliest tumour in each whole group (6 months for MCN, 5 months for MNC), so that in these tables the $F_1$ and $F_2$ incidences are less than the corres-

ponding figures in Table III, where (for comparison between the individual generations) the incidences were based on the numbers alive at 8 and 7 months respectively in the MCN and MNC $F_1$'s, and at 7 and 6 months in the $F_2$'s.

In all 10 generations of each group of injected hybrids the tumour incidences remained high, but with wide fluctuations. In the MCN group (Table XIV) the total incidences were 35·9 per cent in 468 females and 24·3 per cent in 445 males, a significant difference.[32] In the MNC hybrids (Table XV) the incidences were 34·5 per cent in 643 females and 22·2 per cent in 744 males, also a significant difference.[33] The differences between the hybrids were not significant for either sex.

TABLE XIV.—*Incidence of Induced Lung Tumours in Mice of each Generation of MCN Hybrids, Living to 6 Months and Over.*

| Generation. | Sex. | Number of mice. Total in experiment. | Surviving to tumour age. | Tumorous mice. Number. | Percentage. |
|---|---|---|---|---|---|
| $F_1$ | Females | 47 | 44 | 18 | 40·9 |
| | Males | 50 | 49 | 15 | 30·6 |
| $F_2$ | Females | 134 | 111 | 45 | 40·5 |
| | Males | 120 | 99 | 34 | 34·3 |
| $F_3$ | Females | 45 | 44 | 26 | 59·1 |
| | Males | 46 | 45 | 14 | 31·1 |
| $F_4$ | Females | 59 | 54 | 17 | 31·5 |
| | Males | 58 | 47 | 10 | 21·3 |
| $F_5$ | Females | 58 | 52 | 9 | 17·3 |
| | Males | 66 | 56 | 10 | 17·9 |
| $F_6$ | Females | 12 | 11 | 6 | 54·5 |
| | Males | 13 | 6 | 1 | 16·7 |
| $F_7$ | Females | 25 | 23 | 5 | 21·7 |
| | Males | 25 | 20 | 2 | 10·0 |
| $F_8$ | Females | 53 | 44 | 17 | 38·6 |
| | Males | 70 | 56 | 9 | 16·1 |
| $F_9$ | Females | 39 | 35 | 11 | 31·4 |
| | Males | 44 | 31 | 5 | 16·1 |
| $F_{10}$ | Females | 66 | 50 | 14 | 28·0 |
| | Males | 50 | 36 | 8 | 22·2 |
| Total | Females | 538 | 468 | 168 | 35·9 |
| | Males | 542 | 445 | 108 | 24·3 |

In each of the first 2 generations, the tumour incidences in males and females did not differ significantly whether based on the earlier tumour ages in Tables XIV and XV or on the later ages in Table III. The mice lived longer in the earlier than in the later generations. It was proved on further analysis that the sex-difference in each group was due to a greater proportion of males than females dying young. When the figures were corrected, in exactly the same way as shown in Table XII for the NC mice, to what they would have been if the males

TABLE XV.—*Incidence of Induced Lung Tumours in Mice of each Generation of MNC Hybrids Living to 5 Months and Over.*

| Generation. | Sex. | Number of mice. Total in experiment. | Surviving to tumour age. | Tumorous mice. Number. | Percentage. |
|---|---|---|---|---|---|
| $F_1$ | Females | 51 | 48 | 15 | 31·3 |
| | Males | 54 | 51 | 13 | 25·5 |
| $F_2$ | Females | 122 | 121 | 39 | 32·2 |
| | Males | 125 | 125 | 40 | 32·0 |
| $F_3$ | Females | 33 | 33 | 11 | 33·3 |
| | Males | 36 | 36 | 6 | 16·7 |
| $F_4$ | Females | 107 | 105 | 39 | 37·1 |
| | Males | 110 | 104 | 27 | 26·0 |
| $F_5$ | Females | 60 | 58 | 20 | 34·5 |
| | Males | 88 | 82 | 17 | 20·7 |
| $F_6$ | Females | 49 | 46 | 11 | 23·9 |
| | Males | 51 | 48 | 10 | 20·8 |
| $F_7$ | Females | 84 | 83 | 23 | 27·7 |
| | Males | 96 | 93 | 13 | 14·0 |
| $F_8$ | Females | 31 | 29 | 15 | 51·7 |
| | Males | 50 | 50 | 11 | 22·0 |
| $F_9$ | Females | 62 | 57 | 22 | 38·6 |
| | Males | 79 | 76 | 15 | 19·7 |
| $F_{10}$ | Females | 70 | 63 | 27 | 42·9 |
| | Males | 81 | 79 | 13 | 16·5 |
| Total | Females | 669 | 643 | 222 | 34·5 |
| | Males | 770 | 744 | 165 | 22·2 |

had lived to the same ages as the females, it was found that in the MCN group there would have been 161 tumour males or 36·2 per cent, and in the MNC group there would have been 252 tumour males or 33·9 per cent. These figures were in agreement with the incidences in the females in each group.

As shown in Table XVI, tumours were found later in the females than in the males of both groups, the sex-difference being significant. The average ages at death of the non-tumour mice, all of which lived to the lowest tumour age for their group, were very much less than the average ages at death of the tumour mice. There was no sex-difference in age at death of non-tumour mice. A high proportion of non-tumour mice of both sexes died before mean tumour age.

In both groups the incidence of induced tumours was much greater than that of spontaneous tumours and the tumour age was significantly lowered. The lower age may have been due to injected mice coming to autopsy at an earlier age, tumours therefore being seen earlier, or the tumours may have actually been induced earlier.

c. *Multiple nodules.*

All the tumour-bearing mice were classified into 2 groups, those with solitary tumours and those with multiple (two or more) nodules, as observed with the

TABLE XVI —*Average Ages at Death of Tumour and Non-tumour MCN and MCN Hybrids.*

| Strain. | Tumour females. Number. | Tumour females. Average age at death (months). | Tumour females. $\sigma$. | Tumour males. Number. | Tumour males. Average age at death (months). | Tumour males. $\sigma$. | Non-tumour females. Number. | Non-tumour females. Average age at death (months). | Non-tumour females. $\sigma$. | Non-tumour males. Number. | Non-tumour males. Average age at death (months). | Non-tumour males. $\sigma$. | Earliest tumour (months). | Latest tumour (months). |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MCN . | 168 | 15·6 | 4·8 | 108 | 14·1 | 4·7 | 300 | 8·7 | 4·7 | 337 | 8·0 | 3·7 | 6 | 28 |
| MNC . | 222 | 15·6 | 5·6 | 165 | 13·7 | 5·3 | 447 | 7·8 | 3·0 | 579 | 8·0 | 3·4 | 5 | 32 |

naked-eye. The post-mortem records did not always state the exact number of nodules, unless solitary.

In the control groups, Generations 1 to 12 (Table IV), the majority of mice had single tumours, but there were some with 2 and a very few with a greater number. In the injected groups, Generations 1 to 10 (Table IV), the proportions were reversed, the majority of mice having 2 or more nodules and many having " multiple " nodules.

There was no sex-difference in any of the groups except MNC, where 34·2 per cent of tumour females and 47·9 per cent of tumour males had single nodules, the difference of 13·7 per cent being significant.[34] Heston and Deringer (1952) found a greater number of multiple nodules in female guinea pigs.

The differences in numbers of nodules between the reciprocal hybrids were not significant as regards either spontaneous or induced tumours. There was a significant difference of 15·4 per cent between the NC and the uninjected MNC groups,[35] the latter having the greater proportion of solitary tumours ; it has been shown above that the latter group is more resistant. But the uninjected MNC mice also had a significantly greater proportion of solitary nodules (91·7 per cent) than the uninjected MCN mice (77·5 per cent),[36] although the latter were believed to be the more resistant group.

## DISCUSSION.

### 1. *The anomalous $F_1$ result.*

The analysis of lung tumour incidence in the $F_1$ reciprocal hybrids showed no difference between spontaneous and induced tumours. This was contrary to all previous work in this field, which has established beyond doubt that the incidence of lung tumours is increased in mice treated with carcinogens, whether administered by painting, by subcutaneous or by intravenous injection (Lynch, 1927 ; Andervont, 1937*a*, 1939 ; Shimkin, 1940*a*, 1940*b* ; Andervont and Shimkin, 1940 ; Heston, 1940).

Although a number of different matings between the pure strains had been made, half the mice of each $F_1$ litter were injected, so that the contribution of the various matings to the injected and uninjected groups were similar, and there could be no question of the control $F_1$ animals differing from the injected in susceptibility.

It was then discovered that, by what is now realised to have been an error of judgment, but partly to ensure uniformity of environment for treated and untreated mice and also for the compelling reasons of available space and labour, in many instances (but by no means in all) the injected and uninjected $F_1$ mice of the same litter were kept in the same box either throughout their lives or until

transferred to the breeding boxes. This occurred only with $F_1$; in all the other generations the injected and uninjected mice were kept apart.

It is possible that some of the carcinogen, oozing out on to the coats of the injected animals as it is liable to do after subcutaneous injection, was transferred to the uninjected either by skin contact or by licking. The amount may have been small in proportion to that injected; no skin tumours were found in the controls; the average lung tumour age was much greater in the $F_1$ controls (possibly because the latter lived much longer) than in the treated animals; the majority of the control tumorous mice had solitary lung nodules in contrast to the high proportion of multiple nodules in the treated mice. But quite small amounts of carcinogen are known to produce lung tumours by intravenous injection in Strain A mice, and the weaker the dose, the fewer nodules per mouse (Shimkin, 1940*b*; Shimkin and McClelland, 1949). Andervont and Dunn (1953) obtained a few lung tumours in $DBA_f/2$ mice by oral administration of methylcholanthrene. Bagshaw and Strong (1950) did not believe that such quantities as might be ingested in this way could induce tumours of the forestomach, but Andervont and Dunn (1953) discovered that mammary tumours could be induced by oral administration of methylcholanthrene after they had suggested (Andervont and Dunn, 1950) that the licking habits of mice might be responsible for some of their earlier results.

To test the possibility that such licking habits might be the cause of the high $F_1$ incidences in the present work, the incidences of tumours of the forestomach (both papillomata and epitheliomata) were analysed, but these showed no comparable effect. In the untreated $F_1$ reciprocal hybrids the incidence of these tumours was about 14 per cent; in the treated $F_1$ hybrids the incidence was about 60 per cent and the tumours were seen from 7 to 8 months earlier, differences which were significant and implied a definite action by the carcinogen in the treated mice. Gross epitheliomata were found much more frequently in the treated animals. It is intended to make these tumours the subject of a subsequent communication.

Different tissues are known to have different susceptibilities to the same carcinogen (Andervont and Shimkin, 1940; Rask-Nielsen, 1950) and a dose which can induce lung tumours might be ineffective in the case of the forestomach, although it might be thought that oral administration would be more likely to affect the stomach than the lungs. The figures for mammary tumour incidence in the present material are now being reconsidered in the light of the present discovery and of the results of Andervont and Dunn (1953), and may likewise show the effect of the $F_1$ contamination, although a first analysis suggested other causes for the appearance of these tumours.

The conclusion is, therefore, that while it is not certain that any contamination took place in the $F_1$ mice, or that a sufficiently potent quantity of the carcinogen was transferred in the event of such contamination, the possibility of the occurrence does provide an explanation of the anomalous result and must be borne in mind both in the present case and in the analysis of other types of remote neoplasms in the reciprocal hybrids.

2. *Comparison of reciprocal hybrids.*

If the high incidences of lung tumours in " untreated " $F_1$ mice were due to the ingestion of small amounts of methylcholanthrene, it is not possible to state

definitely whether the reciprocal $F_1$ hybrids were alike in susceptibility to spontaneous lung tumours. It seems probable that they were, since previous work has shown that susceptibility to induced tumours parallels that to spontaneous tumours (Andervont, 1937*a*; Shimkin, 1940*a*). Heston (1942*a*) found no difference between reciprocal $F_1$ hybrids (between a susceptible and a resistant strain) as regards induced tumours, and presumably none in regard to spontaneous tumours (Heston, 1942*b*) since both lots of hybrids were combined in one group.

In the present case, the incidences of spontaneous tumours in $F_2$ were less than in $F_1$ in both groups of hybrids. The incidences in later generations diverged, CN mice becoming less susceptible, while the incidence in NC remained fairly high. Heston (1942*a*) and Andervont (1937*b*) both reported $F_2$ to be less susceptible than $F_1$ to induced tumours; this was not observed in the present work, where the two generations showed no significant difference.

The incidence of spontaneous tumours in the early generations of hybrids was higher than in the parent strains, even excluding $F_1$. Andervont (1940) observed the same phenomenon with regard to induced tumours, but the present material did not show it for induced tumours. Although each of the parent strains had a relatively low susceptibility to spontaneous tumours, the incidence of induced tumours in CBA mice was higher than in the reciprocal $F_1$ hybrids but not significantly so. The reciprocal hybrids were equally susceptible to induced tumours; this was shown both by the numbers of tumorous mice and by the percentages with multiple lung nodules. Heston and Deringer (1949) found the data on incidence less striking than the difference in number of nodules.

The fact that, in spite of the suspected contamination of $F_1$, the tumour incidences fell in later generations of CN hybrids and also in the uninjected MCN and MNC mice (descendants of treated animals) confirms the opinion that the effect of the carcinogen was confined to those mice which were exposed to it and that there was no continuing effect on their untreated offspring. Strong (1943) obtained a new line of mice characterised by a high incidence of lung carcinoma after hybridisation (and methylcholanthrene injection in earlier generations) and believed this to be due to a continuing inheritable effect of the carcinogen.

No real sex-difference was observed in the incidence of either spontaneous tumours (as noted also by Shimkin, 1940*b*; and by Heston and Deringer, 1947) or induced tumours (as found also by Andervont, Grady and Edwards, 1942; Heston, 1942*a*; Strong, 1943; and many others). Apparent sex-differences were found to be due to early deaths of the males. For the same reason, the average tumour age was usually earlier for males than females (as noted also by Bittner, 1936).

## SUMMARY.

1. The incidence of spontaneous lung tumours was investigated in the inbred strains of mice, CBA and NBT, and in their reciprocal hybrids, and compared with the incidence of lung tumours induced in the same material by subcutaneous injection of methylcholanthrene.

2. The CBA mice were more susceptible to both spontaneous and induced lung tumours than the NBT mice. There was no difference between the reciprocal hybrids in susceptibility to induced tumours or, in the early generations, to spontaneous tumours. Twelve generations of inbreeding caused genetical

divergence in the 2 groups of hybrids, the CN mice becoming less susceptible to spontaneous lung tumours and the NC mice retaining a moderately high susceptibility. The reciprocal hybrids, in the early generations, were more susceptible than the parent strains to spontaneous tumours.

3. The incidence of lung tumours and the proportion of tumorous mice bearing multiple lung nodules were greatly increased by injection of 1·0 mg. methylcholanthrene. This dose was sufficiently high to obliterate the genetical divergence of the 2 groups of hybrids.

4. The mean tumour age for induced tumours was much less than that for spontaneous tumours. This may have been due to the earlier deaths of treated animals, whereby the tumours were seen earlier, but may also have been due to an acceleration of the process leading to neoplasia.

5. There was no real sex-difference in the incidences of spontaneous or of induced lung tumours. Both types of tumours were found later in females owing to the longer life of the latter.

6. There was no indication of a persistence of the effect of methylcholanthrene in raising the lung tumour incidence. Uninjected generations regained the low incidence of spontaneous tumours characteristic of their group.

7. The effect of a possible contamination of the untreated $F_1$ hybrids with slight amounts of methylcholanthrene is discussed with regard both to lung tumours and other types of remote neoplasms. The possibility of this contamination was discovered when the tumour incidences in injected and uninjected $F_1$ mice were found to be the same.

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## REFERENCES.

ANDERVONT, H. B.—(1937*a*) *Publ. Hlth. Rep., Wash.*, **52**, 212.—(1937*b*) *Ibid.*, **52**, 304.—(1939) *Ibid.*, **54**, 1512.—(1940) *J. nat. Cancer Inst.*, **1**, 135.

*Idem* AND DUNN, T. B.—(1950) *Ibid.*, **10**, 895.—(1953) *Ibid.*, **14**, 329.

*Idem*, GRADY, H. G., AND EDWARDS, J. E.—(1942) *Ibid.*, **3**, 131.

*Idem* AND SHIMKIN, M. B.—(1940) *Ibid.*, **1**, 225.

BAGSHAW, M. A., AND STRONG, L. C.—(1950) *Ibid.*, **11**, 141.

BITTNER, J. J.—(1936) *Amer. J. Cancer*, **27**, 519.

HESTON, W. E.—(1940) *J. nat. Cancer Inst.*, **1**, 105.—(1942*a*) *Ibid.*, **3**, 69.—(1942*b*) *Ibid.*, **3**, 79.

*Idem* AND DERINGER, M. K.—(1947) *Ibid.*, **7**, 463.—(1949) *Ibid.*, **10**, 119.—(1952) *Ibid.*, **13**, 705.

LYNCH, C. J.—(1927) *J. exp. Med.*, **46**, 917.—(1940) *Proc. Soc. exp. Biol. N.Y.*, **43**, 186.

MILLER, E. W., AND PYBUS, F. C.—(1952) *Ann. Rep. Brit. Emp. Cancer Campgn.*, **30**, 205.—(1954) *Brit. J. Cancer*, **8**, 163.

RASK-NIELSEN, R.—(1950) *Ibid.*, **4**, 108.

SHIMKIN, M. B.—(1940*a*) *Arch. Path.*, **29**, 229.—(1940*b*) *Ibid.*, **29**, 239.

*Idem* AND MCCLELLAND, J. N.—(1949) *J. nat. Cancer Inst.*, **10**, 597.

STRONG, L. C.—(1936) *Brit. J. exp. Path.*, **17**, 60.—(1940) *Amer. J. Cancer*, **39**, 347.—(1943) *Arch. Path.*, **36**, 58.—(1945) *J. nat. Cancer Inst.*, **5**, 339.

## NOTES.

1 $\chi^2 = 2{\cdot}9$, $P < 0{\cdot}05$ in CBA. $\chi^2 = 0{\cdot}3$, $P > 0{\cdot}5$ in NBT.
2 $\chi^2 = 10{\cdot}0$, $P < 0{\cdot}01$.
3 $2 \times \sigma d = 4{\cdot}06$; $d = 4{\cdot}125$ in NBT. $2 \times \sigma d = 2{\cdot}104$; $d = 3{\cdot}862$ in CBA.
4 $\chi^2 = 1{\cdot}4$, $P > 0{\cdot}2$.
5 $\chi^2 = 0{\cdot}2$, $P > 0{\cdot}5$.
6 $\chi^2 = 17{\cdot}7$, $P < 0{\cdot}01$.
7 $2 \times \sigma d = 2{\cdot}8$, $d = 3{\cdot}3$.
8 Difference between percentages = 49·2, 2 × standard error = 34·7.
9 $\chi^2 > 9{\cdot}7$, $P < 0{\cdot}01$.
10 $2\sigma d = 3{\cdot}8$, $d = 6{\cdot}3$ in M/CBA. $2\sigma d = 4{\cdot}8$, $d = 2{\cdot}0$ in M/NBT.
11 All CN *v.* CN $F_1$. ♀♀: $d = 1{\cdot}9$, $2\sigma d = 2{\cdot}4$; ♂♂: $d = 1{\cdot}9$, $2\sigma d$ 1·5.
All NC *v.* NC $F_1$. ♀♀: $d = 3{\cdot}3$, $2\sigma d = 2{\cdot}3$; ♂♂: $d = 1{\cdot}0$, $2\sigma d = 1{\cdot}8$.
12 CN $F_1$ ♀♀ *v.* ♂♂: $d = 2{\cdot}7$, $2\sigma d = 2{\cdot}534$.
NC $F_1$ ♀♀ *v.* ♂♂: $d = 5{\cdot}5$, $2\sigma d = 2{\cdot}586$.
13 CN ♀♀ *v.* NC ♀♀: $d = 0{\cdot}1$, $2\sigma d = 3{\cdot}12$.
CN ♂♂ *v.* NC ♂♂: $d = 2{\cdot}7$, $2\sigma d = 1{\cdot}829$
14 $\chi^2 = 9{\cdot}8$, $P < 0{\cdot}01$.
15 $\chi^2 = 5{\cdot}375$, $P < 0{\cdot}05$ and $> 0{\cdot}02$.
16 CN $F_1$ ♂♂ *v.* CN $F_2$ ♂♂: $d = 2{\cdot}1$, $2\sigma d = 1{\cdot}7$.
17 All MCN *v.* MCN $F_1$ (males) $d = 5{\cdot}9$, $2\sigma d = 2{\cdot}4$.
All MNC *v.* MNC $F_1$ (males) $d = 6{\cdot}9$, $2\sigma d = 3{\cdot}3$.
18 MCN $F_2$ *v.* CN $F_2$. $\chi^2 = 15$, $P < 0{\cdot}01$.
MNC $F_2$ *v.* NC $F_2$. $\chi^2 = 12$, $P < 0{\cdot}01$.
19 MCN $F_1$ *v.* $F_2$ (males) $d = 6{\cdot}0$, $2\sigma d = 2{\cdot}7$.
MNC $F_1$ *v.* $F_2$ (males) $d = 4{\cdot}9$, $2\sigma d = 3{\cdot}8$.
20 CN $F_1$ *v.* MCN $F_1$. $\chi^2 > 8$, $P < 0{\cdot}01$. NC $F_1$ *v.* MNC $F_1$. $\chi^2 > 10$, $P < 0{\cdot}01$.
CN $F_2$ *v.* MCN $F_2$. $\chi^2 > 29$, $P < 0{\cdot}01$. NC $F_2$ *v.* MNC $F_2$. $\chi^2 > 9$, $P < 0{\cdot}01$.
21 $\chi^2 > 26$, $P < 0{\cdot}01$.
$\chi^2 > 14$, $P < 0{\cdot}01$ ($F_2$–$F_{12}$).
22 $d = 5{\cdot}9$, $2\sigma d = 0{\cdot}74$ (CN ♀♀ *v.* ♂♂).
23 $d = 1{\cdot}3$, $2\sigma d = 0{\cdot}56$ (uninjected MCN ♀♀ *v.* ♂♂).
24 CN ♀♀ *v.* MCN ♀♀: $d = 5{\cdot}7$, $2\sigma d = 0{\cdot}69$.
CN♂♂ v. MCN ♂♂ $d = 1{\cdot}1$, $2\sigma d = 0{\cdot}62$.
25 $\chi^2 > 10{\cdot}8$, $P < 0{\cdot}01$.
$\chi^2 > 14{\cdot}8$, $P < 0{\cdot}01$ ($F_2$–$F_{12}$).
26 $\chi^2 > 9{\cdot}4$, $P < 0{\cdot}01$.
27 $d = 5{\cdot}9$, $2\sigma d = 0{\cdot}74$.
28 ♀♀: $d = 1{\cdot}8$, $2\sigma d = 0{\cdot}8$. ♂♂: $d = 5{\cdot}0$, $2\sigma d = 0{\cdot}6$.
29 ♀♀: $d = 1{\cdot}6$, $2\sigma d = 1{\cdot}4$: ♂♂: $d = 4{\cdot}7$, $2\sigma d = 1{\cdot}8$.
30 $\chi^2 > 12$, $P < 0{\cdot}01$.
31 $\chi^2 > 8{\cdot}9$, $P < 0{\cdot}01$.
32 $\chi^2 > 10$, $P < 0{\cdot}01$.
33 $\chi^2 > 16$, $P < 0{\cdot}01$.
34 $d = 13{\cdot}7$, 2 × standard error = 9·8.
35 $d = 15{\cdot}4$, 2 × standard error = 7·7.
36 $d = 14{\cdot}2$, 2 × standard error = 13·9.

# THE MORPHOLOGY AND GROWTH CHARACTERISTICS OF A TRANSPLANTABLE MAMMARY FIBROADENOMA IN THE RAT.

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THE successful transplantation of a spontaneous mammary fibroadenoma from a stock female rat and the realization that this type of growth presented many interesting aspects relative to both normal and neoplastic tissue led to a continuation and detailed study of this tumour line. Although the literature is fairly extensive in this field the growth potentialities of these benign tumours, as evidenced by the diversity of reported observations, was believed to justify the undertaking of such work.

In the course of experimentation three integrated but nevertheless distinct branches of research evolved. These are outlined as follows :

(1) The study of the morphology and growth characteristics of this line of mammary fibroadenoma in the course of transplantation.

(2) The determination of the effect of endogenous and exogenous hormones upon the growth characteristics and morphology of these tumours.

(3) A study of the behaviour on transplantation of fibroadenomata which had undergone a fibrosarcomatous transformation and the determination of the extent to which the change to a morphologically malignant form had influenced the response to various hormonal factors known to affect the growth of the benign fibroadenoma.

The results obtained in these studies will be presented in three publications.

The major descriptive works in this field have been presented by the following : Heiman (1934), Heiman and Krehbiel (1936), Robinson and Grauer (1932), Grauer and Robinson (1932 and 1935), Emge (1938), Mohs (1940), Selbie (1941, 1942 and 1950) ; Oberling, Guerin and Guerin (1933, 1935, 1937 and 1938) and Roussy, Guerin and Guerin (1944).

From a consideration of these papers it may be noted that spontaneous and transplanted mammary fibroadenomata in rats are well encapsulated, usually lobulated growths, and pearly white or pinkish in colour. Microscopically these tumours can present the appearance of pericanalicular or intracanalicular fibroadenoma, the former predominating in frequency. The proportion of epithelium (acini and/or ducts) to connective tissue may vary considerably, from the extremes of pure adenoma and fibroma. Carcinoma and fibrosarcoma develop from these essentially benign tumours, the fibrosarcomatous transformation being a relatively common occurrence in transplanted tumours. These fibrosarcomata have been found to bear all the characteristics of malignant growths except the ability to metastasize. Selbie (1942) alone has reported the carcinomatous transformation of transplanted mammary fibroadenomata in rats. Squamous and sebacious metaplasia of epithelial elements and bony and cartilagenous metaplasia of connec-

tive tissue elements have been observed. (Oberling *et al.*, 1935, 1938; Selbie, 1941; Roussy, Guerin and Guerin, 1945.)

It appears that the morphological character of a tumour line can follow two main paths; one, to maintain its definition as a fibroadenoma despite the development of occasional fibrosarcomata; the other, to a progressive loss of epithelium in the course of transplantation with the eventual development of a line of pure fibroma or fibrosarcoma.

Considerable variability in the number of takes, the latent periods and the growth rates of transplanted rat fibroadenomata has been observed by most authors. Compared to common transplanted malignant tumours the fibroadenoma has a slow growth rate and can reach a very large size with no apparent harm to the host. The number of takes in a given series varies. Latent periods range from 4 weeks to over one year. The rate of growth also contains wide extremes and is sometimes irregular.

The variable nature of both the morphology and growth characteristics of the benign mammary fibroadenoma suggests its susceptibility to the environmental conditions of the host. The mammary origin of this type of tumour would also lead one to expect some hormonal influence on its growth and morphology. This has been verified by the findings of most workers in this field, for it is generally agreed that mammary fibroadenomata are more successfully propagated in female rats than in males. Heiman and Krehbiel (1936) noted also that increased takes and accelerated tumour growth initiation and growth rate occurred in castrated males with the reverse in castrated females. Mohs (1940), however, found no differences for tumour growth in intact or castrated males although castration of females produced results similar to those of the above authors. Morphologically these tumours respond to the male environment with a depression of epithelial elements and a predominance of connective tissue. Selbie (1941) observed an increased incidence of intracanalicular fibroadenomata in male rats.

The age of the host can also affect the nature of transplanted fibroadenomata, a fact which lends further evidence to the influence of sex hormones on tumour growth. Heiman and Krehbiel (1936) found that transplanted fibroadenomata grew in young immature females as cellular fibromata, in young females of breeding age as fibroadenomata with pronounced glandular hyperplasia and in old females past litter-bearing age as cystadenomata and papillary cystadenomata. These age differences were not apparent in the male. Emge (1938) reported very few takes in rats under 60 days of age, obtaining maximum takes during the period of sexual maturity and a diminished response in old animals.

The response of the mammary fibroadenoma to pregnancy and lactation seems to parallel that of normal mammary tissue at least with regard to its morphology. Some disagreement arises, however, as to the effect of pregnancy on the rate of tumour growth. Most workers have reported growth acceleration during pregnancy but Emge and Wulff (1934), using more quantitative methods, found no increase in tumour growth rates during pregancy and contended that if anything growth retardation was the trend.

Besides the hormonal environment, the genetic background of the host influences the growth of transplanted fibroadenomata. Species specificity (Heiman, 1934) and increased growth energy in selectively inbred rats (Emge, 1938) have been noted. The age of the tumour tissue itself has also been related to the growth and transplantability of these tumours. Heiman (1934) has noted that

more active growth occurred in tumours 2 or 3 months after implantation and that after 90 days there was increased fibrosis with glandular shrinkage. Oberling *et al.* (1937) noted that after a year the development of fibrosarcoma was almost inevitable. Finally, Heiman (1934) and Selbie (1941) found that the transplantability of a tumour decreased with increasing age.

It appears that these benign growths combine the properties of both neoplastic and normal tissue. Thus abnormal and varied growth potentialities, a tendency to respond to factors affecting normal mammary tissue and the nature of the host environment all contribute to the growth of any one tumour.

## METHODS.

Young adult Sprague Dawley rats were used in the experiments to be described. For each transplant series the rats were of the same age. Uniform and fairly rapidly growing tumours were chosen for transplantation. These were invariably from control female rats. Sections of approximately 0·5 cm. in diameter were cut from the peripheral areas of the parent tumour and were implanted subcutaneously in the axillary and inguinal regions. In the early work each rat received two implants while in the later experiments a single axillary implant was employed. This operation was not performed under asceptic conditions but cleanliness was observed throughout.

After tumour growth was initiated, the tumours were measured weekly with calipers and the length and breadth of the tumour surface were marked on cards. An outline of the tumour was sketched and the area was determined by tracing this outline with a planimeter. In the early experiments, where each rat received two tumour grafts, if both implants grew, the areas of each were summed and the total used for the following determinations.

When the experiment was completed the tumour areas were plotted against time (in days) and growth curves drawn. From these graphs, the times for the tumour to grow from the time of implantation to an area of 0·4 square inches and from 0·4 to 3·0 square inches were determined. These two figures have been designated as the " latent period " and " growth period " respectively.

The rational for these determinations was to obtain a comparable set of figures for the quantitative estimation and comparison of the growth characteristics of the various transplant series and the different groups within series. It was found that at a size of 0·4 square inches the tumours could be measured accurately with calipers and that continued growth was usually assured. The limit of 3·0 square inches for the growth period was quite arbitrary. Since an extension of this period did not change the trend of the results obtained, these values were considered a satisfactory indication of the growth rate of the tumour. Mean tumour latent and growth periods for treated and control groups were compared by the " t " test of Fisher, using a probability of 0·05 as the level of significance. All means will be presented with their standard errors.

Unless a tumour was required for transplantation it was allowed to grow as long as the host was in a healthy condition. The animals were not usually killed, therefore, until the tumours had reached a very large size (up to 600 g.). When a tumour-bearing rat was killed, the tumour was excised, sliced into several sections and examined carefully for changes in texture or general appearance. A sample of tumour tissue from an area resembling the typical fibroadenoma may be seen

in Fig. 1. If the tumour contained areas of atypical tissue, portions of these areas were also kept for sectioning. Such macroscopic changes from the typical fibroadenoma usually indicated transformation to fibroma or fibrosarcoma and may be seen in Fig. 2. For confirmation of the histological report this tissue was usually transplanted into four or five rats and the growth characteristics and morphology of the transplanted growths observed.

It has been the aim of this research to study one tumour line in its course through a series of progressive transplantations and under various experimental conditions. Fig. 3 is an outline of the transplantation history of this tumour line. The sub-lines of fibrosarcomata arising from different generations in the benign tumour are not included. The letter F stands for fibroadenoma. The number following (i.e., F1, F2) indicates the tumour generation. The letters A, B and C separate different transplants series within one generation.

The various transplant series will in future be referred to by the code numbers given here. Double implants were made up to and including F7.

## RESULTS.

### 1. *Morphological variations observed.*

The tumour line which has been studied presented no unusual aspects with regard to morphology. However, certain trends may be noted.

The spontaneous tumour from which this line was initiated contained three distinct forms in the single section observed. These comprised areas of closely packed acini surrounded by strands of connective tissue (Fig. 4), areas of ducts dispersed in a connective tissue stroma (Fig. 5) and finally an area of connective tissue alone. A similar degree of acinar development has not been observed in the transplanted tumours. Epithelium was restricted almost entirely to duct tissue and the connective tissue tended to predominate. What was considered the typical fibroadenoma for this tumour line is shown in Fig. 6. Only slightly greater epithelial development than is shown in this section was observed. The cells of individual ducts proliferated to several cell layers and/or the number of ducts in a given area increased slightly. Variation in the degree of connective tissue development was more extensive. Some tumours contained a few atrophic ducts within a microscopic section while others were classified as pure fibromata.

Transformation to fibrosarcoma (Fig. 7) has occurred in approximately 15 per cent of control tumours and bony and cartilagenous metaplasia has been observed once in a fibroadenoma and fairly often in transplanted fibrosarcomata (Fig. 8).

It has been noted that single tumours are not necessarily morphologically uniform. Different areas will have different degrees of epithelial or connective

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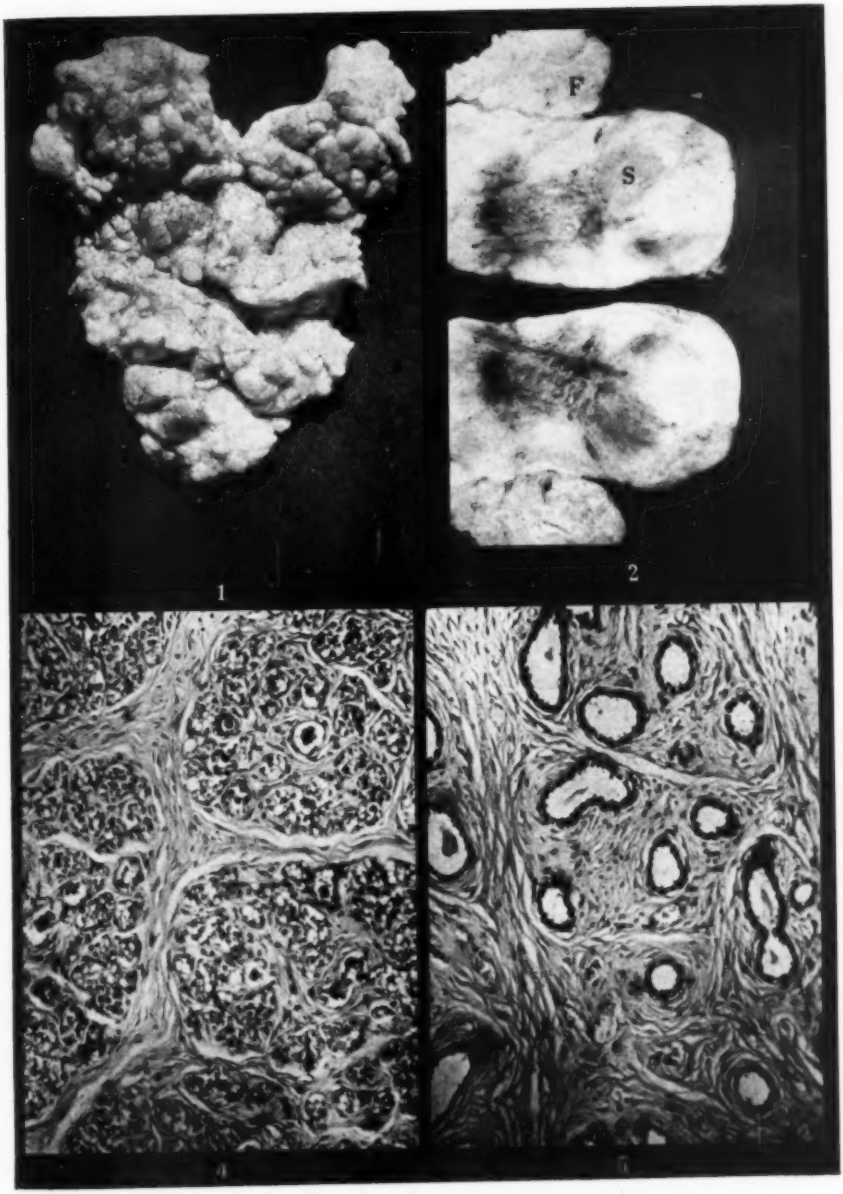
EXPLANATION OF PLATES.

FIG. 1.—Cut surface of transplated fibroadenoma. × ¾.
FIG. 2.—Cut tumour with areas of fibroadenoma and fibrosarcoma. F, fibroadenoma; S, fibrosarcoma. × ¾.
FIG. 4.—Spontaneous mammary fibroadenoma, area of acinar tissue. × 110.
FIG. 5.—Spontaneous mammary fibroadenoma, area of duct tissue. × 110.
FIG. 6.—Typical transplanted fibroadenoma. × 110.
FIG. 7.—Fibrosarcoma originating from fibroadenoma. × 500.
FIG. 8.—Cartilagenous metaplasia of fibrosarcoma. × 110.
FIG. 9.—Fibroadenoma with malignant stroma. × 500.

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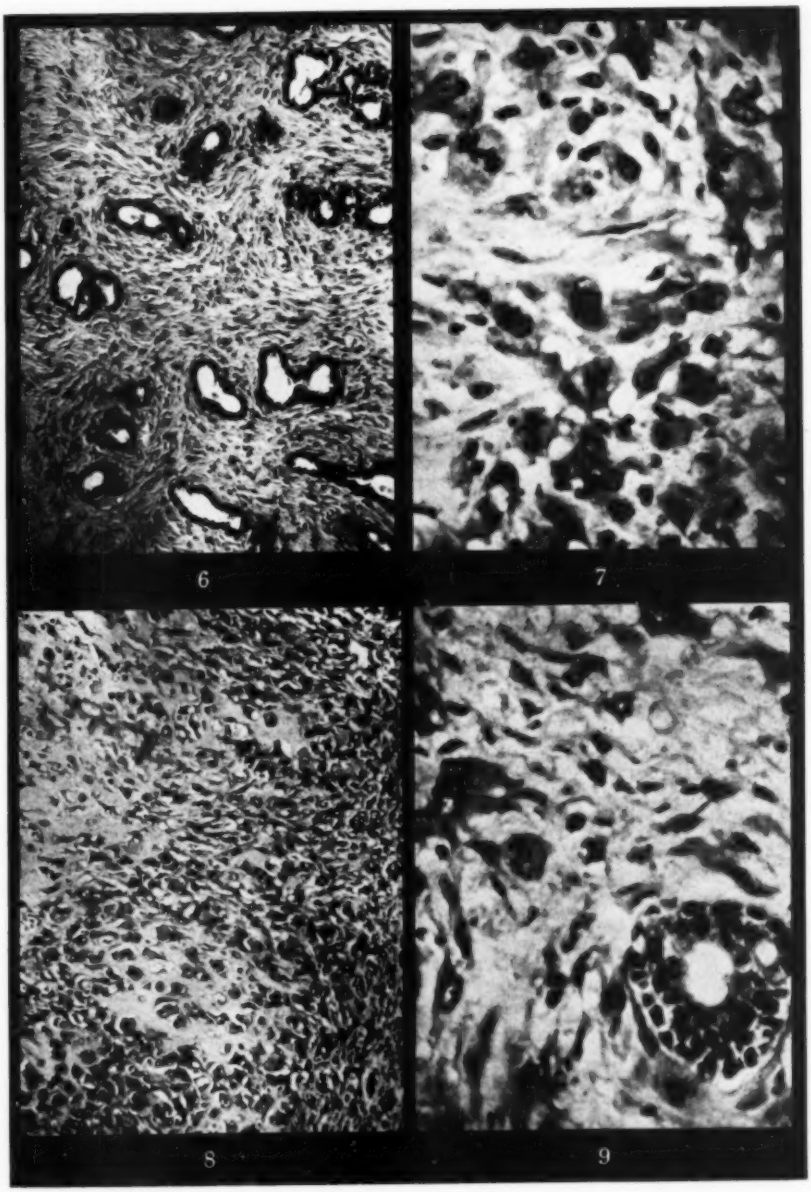

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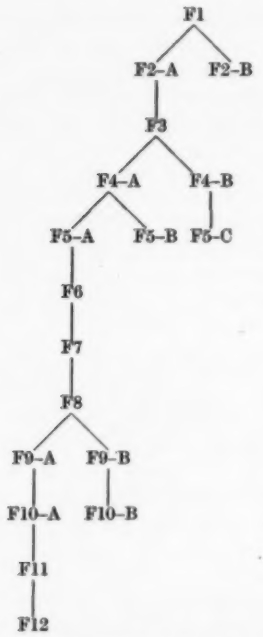


FIG. 3.—Spontaneous tumour (F). The transplantation history of a spontaneous mammary fibroadenoma.

tissue development even within a single microscopic section. Likewise the co-existence of fibrosarcoma and fibroadenoma was more frequently observed than the transformation *in toto* to fibrosarcoma. There was usually a fairly clear line of demarcation between the two adjacent forms, a division which was often detectable on macroscopic examination. The sarcomatous tissue tended to be softer, more translucent and more uniform (Fig. 2). Only one instance has been noted wherein the sarcomatous tissue appeared to infiltrate the portion of the tumour containing epithelium (Fig. 9). The duct cells although hyperplastic did not appear to be involved in the malignant process.

Epithelial or connective tissue development and the incidence of fibrosarcomata varied randomly in the course of twelve generations of transplantation, the tumour line maintaining its identification of benign fibroadenoma. It was not possible to predict the morphology of the tumours in each transplant series from that of the parent tumours. According to the sections observed no extremely fibrous fibroadenomata or fibromata were used for implantation. However, the lack of morphological uniformity in some tumours makes it impossible to be sure of the cellular constitution of the individual implants.

*2. Growth characteristics.*

The criteria for the study of growth in transplanted fibroadenomata are threefold; i.e. the number of tumour takes in a given group of rats bearing tumour

implants, the period of latency before tumour growth is initiated and the actual growth rate of the tumour. The latter two are presented as the "latent" and "growth" periods defined in the section of methods. The range for group size, percentage of tumour takes and the mean latent and growth periods for the tumours of female control groups of all transplant series are given in Table I.

TABLE I.—*Range for Group Size, Tumour Takes and Mean Latent and Growth Periods for Control Female Rats in All Transplant Series.*

| Size of group. | Per cent of tumour takes. | Mean latent period (days). | Mean growth period (days). |
|---|---|---|---|
| 5 to 20 | 25 to 100 | $37 \pm 4{\cdot}4$ to $112 \pm 25{\cdot}1$ | $27 \pm 1{\cdot}1$ to $96 \pm 23{\cdot}2$ |

In the course of twelve generations of transplantations no indication of an increase or decrease in growth potentialities was evident. The parent tumours had latent and growth periods ranging from 25 to 64 and 25 to 60 days respectively. The age of these tumours varied from 59 to 164 days from the time of tumour implantation to the death of the host. Within the limits observed there did not appear to be any obvious correlation between the growth characteristics or age of the parent tumour with the growth characteristics of the resulting transplants.

3. *Relation of tumour morphology to growth characteristics.*

It became apparent while observing this tumour line that for a series in which tumour growth was relatively uniform and rapid, the morphology tended also to have a narrow range of variation. Alternately, a wide variation in growth characteristics was accompanied by a comparable degree of morphological variation. Although not without exception, slow growing tumours tended to be more fibrous.

Most marked and more easily demonstrated was the difference in the development of fibrosarcomata in relatively fast and slow growing tumours. Table II gives the mean latent and growth periods for the tumours which remained benign and those which became sarcomatous in control female rats. Both periods are longer for the latter group and the difference is highly significant ($P < 0{\cdot}01$)

TABLE II.—*Mean Latent and Growth Periods for Benign and Malignant Control Tumours.*

| Tumour. | No. of rats. | Mean latent period (days). | Mean growth period (days). |
|---|---|---|---|
| Benign | 38 | $53 \pm 3{\cdot}1$* | $51 \pm 5{\cdot}2$* |
| Malignant | 10 | $104 \pm 12{\cdot}3$ | $125 \pm 20{\cdot}1$ |

* $P < 0{\cdot}01$—Benign versus malignant tumours.

These data show that the fibrosarcomatous transformation was limited to tumours in which growth initiation and/or growth rate was retarded. Short latent periods and rapid growth, on the other hand, appeared to ensure benignancy and reduce morphological variation within the benign form.

4. *Endogenous hormonal factors affecting the growth and morphology of transplanted mammary fibroadenomata.*

Experiments have been conducted to determine the effect of sex, ovariectomy and pregnancy on transplanted mammary fibroadenomata in rats. These results tended to confirm the findings reported in the literature. In this initial paper, however, the following growth data will be presented, not only to give evidence for the responses claimed but also to demonstrate the inconsistency of response manifestation for the three criteria used (tumour takes, latent periods and growth periods). It has been observed that in individual experiments the effect was seldom apparent for all three criteria and that in different series this effect was not always expressed by the same criterion. Because of this, the pooling of data from several series to present a single mean figure for each determination would in most cases eliminate any indication of a response.

In assessing our results on tumour growth, emphasis has been placed on changes in the latent and growth periods. Variation in tumour takes was not considered very dependable for the comparison of small groups, except where marked differences occurred. Since the data for individual series have not been pooled, representative samples of the results obtained will be presented.

(1) *Effect of Sex.*—Both males and females were implanted with tumour tissue in four series (F4–A, F8, F9–A and F9–B). Evidence of inhibited tumour growth in the male rats was present in all. Table III gives the results obtained for series F9–A and F9–B.

TABLE III.—*Effect of Sex on the Growth of Transplanted Mammary Fibroadenomata.*

| Series. | Sex. | No. of rats. | No. of takes. | Mean latent period (days). | Mean growth period (days). |
|---|---|---|---|---|---|
| F9–A | M | 10 | 8 | 58 ± 6·5* | 63 ± 11·7* |
| | F | 10 | 7 | 41 ± 3·6 | 35 ± 2·0 |
| F9–B | M | 10 | 0 | — | — |
| | F | 10 | 7 | 66 ± 5·9 | 67 ± 13·1 |

* $P < 0{\cdot}05$, males versus females.

Transplanted fibroadenomata in male rats showed a distinct tendency toward the development of more fibrous fibroadenomata and fibromata. In only one series (F8) was there an increased incidence of fibrosarcomata (males—6 tumours, 3 fibrosarcomata ; females—6 tumours, 0 fibrosarcomata).

(2) *Effect of ovariectomy.*—In five series, female rats were ovariectomized 1 or 2 days before implantation of tumour tissue (F4–B, F6, F10–B and F11). In all but one of these experiments there was indication of tumour growth inhibition in the ovariectomized group. Table IV gives the results obtained for Series F8 and F11.

TABLE IV.—*Effect of Ovariectomy on Growth of Transplanted Mammary Fibroadenomata in Rats.*

| Series. | Treatment. | No. of rats. | No. of takes. | Mean latent period (days). | Mean growth period (days). |
|---|---|---|---|---|---|
| F8 | Ovariectomy | 10 | 9 | 48 ± 5·5 | 37 ± 1·5* |
| | Controls | 10 | 10 | 37 ± 4·4 | 27 ± 1·1 |
| F11 | Ovariectomy | 10 | 0 | — | — |
| | Controls | 10 | 7 | 61 ± 6·2 | 56 ± 21·9 |

* $P < 0{\cdot}01$, ovariectomy versus controls.

Ovariectomy of female rats did not appear to affect the morphology of benign tumours or the incidence of fibrosarcomata.

(3) *Effect of pregnancy.*—The experimental work has not been extensive enough to draw any definite conclusions. Groups in series F4–B and F5–B were bred after tumour implantation and in most cases gestation occurred during the latent period, and continuing in a few cases for 1 or 2 weeks of the growth period. A trend to growth stimulation in Series F4–B was not confirmed in the later series. The morphology of these tumours was not studied during pregnancy. No unusual morphological aspects were observed in tumours examined 3 weeks or more after parturition.

## DISCUSSION.

The tumour line observed does not have any unusual morphological or growth characteristics except possibly a limited ability to develop epithelium. The relationship of morphology and growth characteristics, however, may be discussed, particularly with a view to understanding the conditions associated with the fibrosarcomatous transformation of these benign tumours. This event was apparently limited to tumours with extended latent periods and/or slow growth rates. Three possible interpretations can be derived from these findings :

(1) That arrested or slow growing tumours are depressed by some factor intrinsic in the tumour cells or imposed by the internal environment of the host and that the malignant potentialities of the fibroblasts are exerted to overcome this restraining influence.

(2) That the fibrosarcomatous transformation is a slow process and is completed only in retarded or old tumours which allow the prolonged survival of the host.

(3) That the advancing age of the host may produce environmental conditions more favourable for the sarcomatous transformations.

The first interpretation is in accordance with the theory summarized by Haddow and Robinson (1937) that the precancerous state of a cell is one of inhibition of normal growth. The fact that tumour growth inhibition in males and ovariectomized females occurred without an increased incidence of fibrosarcomata does not necessarily contradict this theory since tumour growth in these groups, although slower than female controls, was usually more rapid than that associated with the fibrosarcomatous transformation.

Other workers have observed a high incidence of fibrosarcomata in "old" tumours. (Heiman, 1934 ; Oberling *et al.*, 1937 ; Selbie, 1941). However, if time or tumour age were the only factors involved one would expect that with continued transplantations the development of a line of pure fibrosarcoma would be inevitable. Such a changeover has been found to occur in some cases but the maintenance of the benign form appears equally prevalent.

The changing internal environment of the host with advancing age cannot be ruled out as a factor contributing to this change, for although Heiman and Krehbiel (1936) found that tumour tissue transplanted in old females grew as cystadenomata, the response of newly initiated growths to environmental conditions may not be comparable to that of already established growths.

Selbie (1941) has proposed that the malignant potentialities of the fibroblasts are depressed by epithelial tissue and are exerted only after partial or complete degeneration of the latter. Despite a trend toward the increased incidence of

fibrosarcomata in the more fibrous fibroadenomata, exceptions are too numerous in our observations to substantially support this theory.

It is apparent that no definite conclusions can be drawn with regard to the factors predisposing the fibrosarcomatous transformation of transplanted mammary fibroadenomata. The diversity of observations and the variety of possible interpretations emphasizes the necessity for careful scrutiny of all available data in this field. The association of depressed growth with the fibrosarcomatous transformation has not been considered by other workers.

The hormonal control of mammary fibroadenomata will be discussed more fully in following papers describing the effect of exogenous hormones on tumour growth and morphology. It appears that endogenous estrogen is essential for optimum tumour growth. Our findings would indicate that the male sex hormone actively depresses tumour epithelium since there was a prevalence of more fibrous tumours in male rats but not in ovariectomized females. Heiman (1934), however, reported epithelial depression in the tumours of ovariectomized rats. These observations serve to demonstrate further the differences which can occur for different tumour lines and different strains of rats. Epithelial development of the tumours reported by Heiman (1934) was more extensive and appeared to be more sensitive to variations in hormonal environment than that of the tumours observed in this laboratory.

## SUMMARY

(1) Literature describing the morphology and growth characteristics of transplanted mammary fibroadenomata in rats is reviewed.

(2) A method for the presentation of quantitative data for tumour growth is described. Three criteria are used to assess a response: the number of tumour takes, the latent period and tumour growth rate.

(3) The morphology and growth characteristics of a line of mammary fibroadenomata in rats in the course of twelve generations of transplantation are outlined. Morphological variations included changes in the proportion of epithelium to connective tissue, the development of fibromata and fibrosarcomata and bony or cartilagenous metaplasia. Epithelial development appeared somewhat restricted.

The tumour line has maintained its identity as mammary fibroadenoma and has shown no trend to an increase or decrease in growth potentialities although variation was extensive.

(4) It has been shown that the development of fibrosarcoma was prevalent in tumours in which growth initiation was retarded and/or growth rate slow. Rapid and uniform growth was usually associated with a narrowed range of morphological variation and the maintenance of the benign form. The epithelium tended to be more extensive in such tumours.

(5) The effect of endogenous hormones was demonstrated by a retardation of tumour growth in males and ovariectomized females. Epithelial development was reduced in the tumours of male rats but not in ovariectomized females.

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## REFERENCES.

EMGE, L. A.—(1938) *Arch. Path.*, **26**, 429.
*Idem* AND WULFF, L. M. R.—(1934) *West. J. Surg.*, **42**, 45.
GRAUER, R. C., AND ROBINSON, G. H.—(1932) *Amer. J. Cancer*, **16**, 191.—(1935) *Arch. Surg.*, **31**, 677.
HADDOW, A., AND ROBINSON, A. M.—(1937) *Proc. Roy. Soc.*, B., **122**, 422.
HEIMAN, J.—(1934) *Amer. J. Cancer*, **22**, 497.
*Idem* AND KREHBIEL, F.—(1936) *Ibid.*, **27**, 450.
MOHS, F. E.—(1940) *Ibid.*, **38**, 212.
OBERLING, C., GUERIN, M., AND GUERIN, P.—(1933) *Bull. Ass. franç. Cancer*, **22**, 606.—(1935) *Ibid.*, **24**, 232—.(1937) *Ibid.*, **26**, 483.—(1938) *Ibid.*, **27**, 260.
ROBINSON, G. H., AND GRAUER, R. C.—(1932) *Amer. J. Cancer*, **16**, 184.
ROUSSY, G., GUERIN, M., AND GUERIN, P.—(1944) *Bull. Acad. Méd., Paris*, **128**, 156.—(1945) *Ibid.*, **129**, 417.
SELBIE, F. R.—(1941) *Brit. J. exp. Path.*, **22**, 156.—(1942) *Ibid.*, **23**, 61.—(1950) *J. R. micr. Soc.*, **70**, 201.

# EFFECTS OF EXOGENOUS HORMONES ON GROWTH CHARACTERISTICS AND MORPHOLOGY OF TRANSPLANTED MAMMARY FIBROADENOMA OF THE RAT.

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THE description of a line of transplanted mammary fibroadenomata in rats was presented in a preceding paper from this laboratory (Millar and Noble, 1954). Observations by us and others have shown that this type of tumour is more easily propagated, grows more rapidly and usually shows greater epithelial development in the female host than in the male. In addition, castration in males and females increases and decreases respectively the growth energy of transplanted mammary fibroadenomata in rats. In order to elucidate further the nature of the hormonal control of these benign tumours, experiments have been carried out to determine the effect of administered pituitary, adrenal and sex hormones on their growth and morphology.

The effect of estrogens on the growth of mammary fibroadenomata in rats has been studied by several workers with somewhat conflicting results. Heiman and Krehbiel (1936) found no consistent tumour response to administered estrogens. However, estrogen (theelin) plus growth hormone (Antuitrin S) or gonadotrophic hormone (Antuitrin G) increased the number of tumour takes in intact rats, although none of the individual preparations were active. Antuitrin S alone was effective in increasing the takes in castrated males and females, while estrogen and growth hormone were again effective only when injected simultaneously. In these experiments tumours of the 13th to 22nd generations were used. Estrogen administration to rats bearing auto- and homo-transplants of spontaneous tumours produced marked tumour epithelial proliferation and increased growth rate (Heiman, 1940*b*).

Mohs (1940) found that injections of estrogen to castrated rats implanted with fibroadenoma tissue, increased the number of takes in both sexes and increased the tumour growth-rate in female castrates. The tumour lines used by this author were in the first to third transplanted generation. These results, reviewed with those of Heiman *et al.*, suggest that the fibroadenoma may become less responsive to exogenous estrogens in the later generations of a tumour line.

Emge and co-workers found no change in the growth characteristics or morphology of transplanted fibroadenomata with long continued administration of from 50 i.u. bi-weekly to 200 i.u. daily of theelin (Emge, Murphy and Shilling, 1938; Murphy, Shilling and Emge, 1938; Emge and Murphy, 1938). However, larger

doses—up to 1000 i.u. of theelin daily—administered for shorter periods, produced progressive hyperplastic changes in the epithelium of the tumour, ending in fibrosis (Emge, 1939). The aim of the work reported in these papers was to determine whether excess estrogen would be instrumental in initiating a malignant change in the epithelium of the benign mammary fibroadenomata in rats. No such effect was indicated.

Heiman (1940*a*, 1943) has reported that administered testosterone proprionate or progesterone caused a marked reduction of tumour takes and a depression of epithelial development. Testosterone also decreased the tumour growth-rate. These two hormones injected simultaneously effected a greater reduction of takes than either alone. Pregnancy or simultaneous injections of estrogen could offset the inhibitory action of either progesterone or testoterone. Mohs (1940) found no growth inhibition of transplanted fibroadenomata in castrated rats receiving small doses of testosterone, but later reported (Mohs, 1941) that such tumours changed to pure fibromata after 25 weeks of treatment.

## METHODS.

The transplantation technique and procedure for obtaining and evaluating the results on tumour growth are given in detail in the first paper (Millar and Noble, 1954). In brief, tumour areas, determined weekly, were plotted against time to obtain growth curves. Three criteria were used to demonstrate a response, i.e., the number of tumour takes, the latent period and the tumour "growth-rate." The latent period was described as the time lapse between the tumour implantation date and the time the implant had reached a size of 0·4 square inches. An arbitrary "growth period" designated as the time for the tumour to grow from 0·4 to 3·0 square inches served to describe the growth rate. Mean tumour latent and growth periods for treated and control groups were compared by the "t" test of Fisher, using a probability of 0·05 as the level of significance. All means will be presented with their Standard Errors

The tumour line (F) described in the previous paper was used for most of the work to be outlined. Two additional lines (3 F and 4 F) have been used in a few experiments. The transplant generation is designated by the number following the letter F (i.e., 3 F3). Young adult Sprague-Dawley rats were used throughout. The age of rats for each transplant series was the same.

In general, two treatment regimes were used. Unless treatment was started after tumour growth initiation, rats were injected during the first, third and fourth, or fourth and fifth weeks after tumour tissue implantation, or continuously from the implantation date until the death of the animal. The former will be called "limited", the latter "continuous" treatment. Limited treatment was employed in the early work with the F tumour line. This regime was based on an expected latent period of approximately 4 weeks, and the assumption that the first weeks' treatment would affect the establishment of tumour implant and the latter 2 weeks treatment, the initiation of growth and possibly the growth rate. However, the occurrence of tumours with prolonged latent periods made this plan unfeasible and continuous treatment was adopted for later experiments.

The results will be presented in separate sections dealing with the various hormone preparations used for treatment, and particulars regarding dose levels, etc., will be included in each.

## RESULTS.

(1) *Effect of estrogens.*

The effect of estrogens was determined for " low "(1 to 10 $\mu$g. daily) and " high" (50 to 200 $\mu$.g daily) dose levels. In all but one experiment, where estradiol benzoate was used, the synthetic estrogen diethylstilbestrol was employed.

(a) *Low dose levels.*—Seven individuals experiments were carried out to determine the effect of low doses of estrogen on the growth of transplanted mammary fibroadenomata in female rats (Series F3, F4–B, F5–A, F5–C, F6, 3 F3 and 4 F3). " Limited " treatment was employed in all but Series 3 F3 and 4 F3. The estrogen was injected subcutaneously in a 5 or 10 per cent ethyl alcohol solution. Tumour growth stimulation by estrogen was indicated in Series F3 only, since the number of takes was higher and the latent period significantly decreased.

Table I shows results for Series F3 and F6, the latter being representative of the negative groups.

TABLE I.—*Effect of Low Doses of Diethylstilbestrol on the Growth of Transplanted Fibroadenomata in Female Rats.*

| Series. | Treatment. | Daily dose ($\mu$g.). | Number of rats. | Number of takes. | Mean latent period (days). | Mean growth period (days). |
|---|---|---|---|---|---|---|
| F3 | Stilbestrol, limited | 5 | 6 | 6 | 37±2 ·6* | 42±6·0 |
| | Controls | — | 6 | 3 | 60±10·3 | 39±1·8 |
| F6 | Stilbestrol, limited | 10 | 8 | 7 | 27± 2·7 | 41±1·1 |
| | Controls | — | 10 | 9 | 43±15·4 | 35±3·2 |

* Treated versus controls, $P < 0{\cdot}05$.

Negative tumour response occurred despite alterations in the dose level (1·5 and 10 $\mu$g. daily), the type of estrogen used, diethylstilboestrol or estradiol benzoate, and the duration of treatment.

Estrogen treatment did not appear to alter the morphology of the fibroadenomata in female rats.

The effect of small doses of estrogen on the growth and morphology of these benign tumours was studied also for male rats, using both limited and continuous treatment. (Series F4–A, F8 and F–9–A.) Table II gives the growth results obtained.

Although the results in Series F4–A did not differ significantly from the male controls, there was an indication of response in the shorter mean latent period of treated rats. This is more obvious when the individual values are observed for this determination. They were for treated and control tumours, 86, 110, 89 and 219, 136, 133 days respectively. The high degree of significance obtained for the difference between treated and control males in Series F8 confirms the earlier indications. The trend is again evident in Series F9–A, although the results were not statistically significant.

Histologically, enhanced epithelial development was evident in the tumours of continuously treated rats. The majority of these tumours presented the appearance of the " typical " fibroadenoma as described by Millar and Noble (1954). In untreated males the connective tissue stroma was more predominant.

TABLE II.—*Low Doses of Diethylstilbestrol on the Growth of Transplanted Mammary Fibroadenomata in Male Rats.*

| Series. | Treatment. | Number of rats. | Number of takes. | Mean latent period (days). | Mean growth period (days). |
|---|---|---|---|---|---|
| F4–A | Stilbestrol, limited* | 8 | 3 | 95± 7·5 | 105±16·6 |
| | Control, male | 8 | 3 | 163±28·2 | 76± 6·0 |
| | Control, female | 8 | 6 | 64±14·0 | 95±29·6 |
| F8 | Stilbestrol, continuous† | 10 | 8 | 32± 0·7‡ | 24± 0·7‡ |
| | Control, male | 10 | 10 | 45± 3·4 | 32± 1·6 |
| | Control, female | 10 | 10 | 37± 4·4 | 27± 2·7 |
| F9–A | Stilbestrol, continuous* | 10 | 7 | 43± 4·4 | 59±15·6 |
| | Control, male | 10 | 8 | 58± 6·5 | 63±11·7 |
| | Control, female | 10 | 7 | 41± 4·6 | 35± 2·1 |

* Stilbestrol, 5 μg. daily.
† Stilbestrol, 10 μg. every second day.
‡ $P < 0·01$, treated versus male controls.

(*b*) *High doses of Estrogen.*—In the experiments to be described, diethylstilbestrol was administered by subcutaneous injection dissolved in sesame oil or orally as a constituent of the drinking water (0·5 mg. per cent solution). Early experiments (Series F4–B and F6), using the " limited " treatment period, indicated that a daily dose of 100 μg. of diethylstilbestrol (by injection) could delay tumour growth initiation, although significant increases in the mean latent periods were not observed. The trend was substantiated by following experiments in which the treatment period was extended until after tumour growth was well established in the control groups (Series F9–A, F12, 4 F3). For both males and females, no tumour growth occurred during the treatment period and for some weeks after injections were discontinued. Table III gives the data for a few representative experiments.

TABLE III.—*Effect of High Doses of Diethylstilbestrol Injected from the Time of Tumour Implantation on the Growth of Transplanted Fibroadenomata in Rats.*

| Series. | Treatment. | Sex. | Number of rats. | Number of takes. | Mean latent period (days). | Mean growth period (days). |
|---|---|---|---|---|---|---|
| F9–A | Stilbestrol* | F | 10 | 7 | 123± 4·3‡ | 49± 7·0 |
| | Controls | F | 10 | 7 | 41± 3·6 | 35± 2·0 |
| | Stilbestrol* | M | 10 | 6 | 151±10·6‡ | 53± 6·4 |
| | Controls | M | 10 | 8 | 59± 6·5 | 63±11·5 |
| F12 | Stilbestrol† | F | 7 | 4 | 101± 7·7‡ | 38± 3·2 |
| | Controls | F | 10 | 7 | 42± 6·1 | 39± 3·9 |

* Stilbestrol, 400 μg. every 2nd day for 52 days after tumour implantation.
† Stilbestrol, 200 μg. every 2nd day for 58 days after tumour implantation.
‡ $P < 0·01$, treated versus controls.

When administered orally in the drinking water, the daily consumption of diethylstilbestrol was approximately 50 μg. In neither females or males was this amount sufficient to delay the initiation of tumour growth. However, for the females (Series F6) tumour growth was arrested or slowed in 5 of 8 tumour-

bearing rats. The remaining 3 tumours appeared unaffected. The mean growth-period for this group was significantly longer than the controls.

The growth results for male rats were less easily evaluated. Two groups of 8 rats in the same series (Series F8) received diethylstilbestrol orally. One of these groups received, in addition, injections of a crude beef anterior pituitary preparation (to be described in a later section). Only 2 out of 8 tumours in the group receiving stilbestrol alone showed growth retardation, while the growth rate was markedly reduced in 6 of 8 tumours of the group receiving both estrogen and the anterior pituitary preparations. Since, as it will be shown, the anterior pituitary preparation alone can stimulate the growth of mammary fibroadenomata in male rats, it was considered unlikely that this product produced an additive inhibiting action with high doses of diethylstilbestrol. Rather, in view of this consideration and the apparent all or none action of diethylstilbestrol when administered at this dose level, the unequal response of these two groups has been attributed to change variation alone. Therefore, the results from these two groups have been pooled and presented as a single group. In this case the mean growth period of the tumours of treated rats was significantly longer than the controls, as may be seen from Table IV.

TABLE IV.—*Effect of High Doses of Diethylstilbestrol administered Orally from the Time of Tumour Implantation on the Growth of Transplanted Fibroadenomata in Rats.*

| Series. | Treatment.* | Sex. | Number of rats. | Number of takes. | Mean latent period (days). | Mean growth period (days). |
|---|---|---|---|---|---|---|
| F6 | Stilbestrol (continuous) | F | 8 | 8 | 31 ± 3·4 | 113 ± 25·4‡ |
| | Controls | F | 10 | 9 | 43 ± 15·4 | 35 ± 3·2 |
| F8 | Stilbestrol† | M | 20 | 16 | 51·5 ± 10·0 | 79·4 ± 13·6‖ |
| | Controls | M | 10 | 10 | 45·0 ± 3·4 | 32·0 ± 1·6 |

* Drinking water 0·5 mg. per cent diethylstilbestrol, approximate daily intake = 50 μg.
† Stilbestrol, administered for 168 days after tumour implantation.
‡ $P < 0·01$, treated versus controls.
‖ $P < 0·05$, treated versus controls.

Finally, experiments were undertaken to determine the effect of high doses of estrogen on already growing tumours. Rats in two series (F5–B and F12) were injected with stilbestrol after the tumours had reached a size of approximately 0·5 square inches. Treatment for 3 weeks (100 μg. stilbestrol daily) caused little change in tumour growth rate (Series F5–B). In Series F12 tumour-bearing rats were injected every second day for 60 days with 200μg. of diethylstilbestrol. Tumour growth was arrested in 4 rats, while there was no response in the remaining 3. Since some of these animals died before the tumours reached 3 square inches, it was not possible to calculate a mean growth period for this group. The results are presented, therefore, in Fig. 1, which shows tumour areas plotted against time for each tumour in the treated group. As the retarded tumours did not exhibit a rapid recovery after the cessation of treatment, it cannot be ruled out that the tumour growth trend was independent of the treatment. However, no such slow growing tumours occurred in the controls (mean growth period 39 ± 3·9 days) or any other group in this series, a fact which favours the

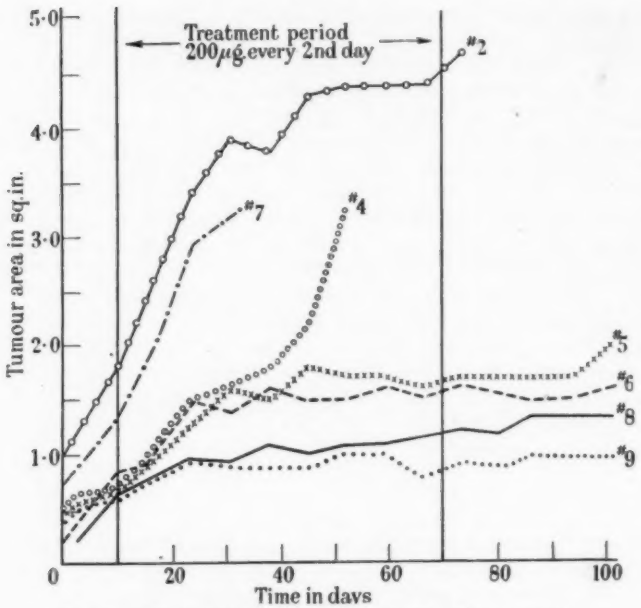


FIG. 1.—Effect of high doses of diethylstilbestrol on growing fibroadenomata in rats (Series F12).

interpretation that the estrogen was at least partially responsible for the depressed growth of these tumours.

Estrogens administered at the high dose levels given cause considerable body growth retardation and occasionally incur the death of the animal. It was necessary, therefore, to determine whether tumour growth inhibition was merely concurrent with body growth depression or whether it could be attributed more specifically to the estrogen administered. It has been reported (Millar and Noble, 1952) that dietary restriction sufficient to cause body growth depression equal to that observed in stilbestrol treated rats (100 μg. daily) had little or no effect on the growth initiation of fibroadenoma implants, or on the tumour growth rates. In addition, the initiation of tumour growth after cessation of stilbestrol injections (treatment started at time of tumour implantation) could not be delayed further by dietary restriction, sufficient to prevent the host from recovering from the body-weight depressing effects of this hormone.

There did not seem to be any alteration in the distribution of the benign variants of the fibroadenoma in animals treated with high doses of stilbestrol. The incidence of fibrosarcomata was unaltered also in these groups, where tumour growth initiation did not occur till after treatment was discontinued. However, for those animals which received stilbestrol during tumour growth there was an increase in the number of sarcomatous transformations, this event being restricted to tumours whose growth had been arrested or retarded during the treatment. The results for female rats are given in Table V.

TABLE V.—*Effect of High Doses of Diethylstilbestrol Administered During Tumour Growth on the Incidence of Fibrosarcomata in Female Rats.*

| Group. | Benign tumours. | Fibrosarcomata. |
|---|---|---|
| Treated (13 rats) | 5 | 8 |
| Controls (19 rats) | 17 | 2 |

$\chi^2$ test, $P < 0{\cdot}01$.

According to the $\chi^2$ test there is a significant difference in the incidence of fibrosarcomata in the treated and control groups. This data includes the groups receiving stilbestrol by injection after tumour growth initiation and those receiving diethylstilbestrol orally from the time of tumour implantation. The latter groups are included since the treatment period extended well after tumour growth initiation.

With regard to male rats on this regime (Series F8) the results were less conclusive. Eight of 16 tumours became sarcomatous in the treated group, and again, this change was restricted to those tumours showing retarded or arrested growths. However, 3 of 6 tumours in the controls became sarcomatous. It would appear, therefore, that tumours in the male rats of this series were initially more predisposed to become fibrosarcomata, since in the control group the sarcomatous tumours were fairly fast growing and showed a tendency to fibrous tissue development while in the benign state.

(2) *Effect of progesterone.*

Experiments were conducted with female rats in which progesterone was injected alone (5 mg. daily) or with low doses of estrogen for the " limited " treatment period (Series F4–B and F5–C). No change was observed in the growth characteristics or morphology of the tumours of treated rats.

(3) *Effect of androgen.*

Testosterone proprionate injected into female rats for the " limited " treatment period (1 mg. daily) did not affect the growth or morphology of fibroadenoma implants (Series F4–B and F5–C). However, when the duration of treatment was extended (106 days after tumour implantation) there was an indication of slight tumour growth inhibition (Series F10–A and F11). Due to the death of some animals before the tumours had reached 3·0 square inches, mean growth periods could not be calculated.

Data regarding tumour morphology was scant, but there was a trend towards increased fibrous tissue development in the tumours of testosterone treated rats.

The results in this section are inadequate for any independent conclusions to be drawn. However, when considering the findings of Heiman and Mohs for the action of androgens on the fibroadenoma which were outlined earlier, the trends observed may be significant.

(4) *Effect of cortisone.*

One experiment in Series F5–B was carried out to determine the effect of cortisone on transplanted fibroadenomata in female rats. Cortisone acetate in aqueous suspension was injected for a period of 3 weeks at a dose level of 5 mg.

daily. The treatment was started after the tumours had reached an initial size ranging from 0·6 to 1·4 square inches. All tumours reached a size of 3·0 square inches before the treatment period was completed. There was no difference in the mean growth periods of treated and control groups. Cortisone treatment caused considerable body weight loss, showing again that body growth depression need not slow the growth of transplanted fibroadenomata. Cortisone treated rats were not killed for 4 or more weeks after injections were discontinued. The tumours showed a normal morphology at this time.

(5) *Effect of anterior pituitary preparations.*

Three pituitary preparations were tested for their effect on the growth and morphology of the transplanted fibroadenoma. These were :

(1) a bovine anterior pituitary saline suspension ;
(2) a sheep pituitary extract ;
(3) a biologically pure growth hormone preparation obtained from Armour Laboratories Inc.

(1) The anterior lobes were dissected from frozen whole beef pituitaries. The required amount of anterior pituitary substance was weighed, ground with sand in a mortar and mixed with physiological saline in the proportions of 1 g. of anterior pituitary to 5 ml. of physiological saline. This was mixed well, centrifuged and the supernatant plus the fine sediment at the top of the residue taken off and refrigerated. No more than a week's supply was prepared at one time. This material was injected subcutaneously in doses of 0·5 ml. daily or 1·0 ml. every second day, this being equivalent to 100 and 200 mg. respectively of anterior pituitary substance.

Biological assays have indicated the presence of growth hormone and prolactin but little gonadotrophic activity in this preparation.

(2) A sheep pituitary extract was prepared in this laboratory over 15 years ago with the purpose of obtaining prolactin. No details, however, are available for the method of extraction. The product was a crude one and it was considered that potentially it contained the activity of many of the pituitary hormones. Biological essays indicated that there was prolactin but no growth hormone activity in the extract. Gonadotrophic activity was not determined. The material was injected subcutaneously in a neutralized water suspension at a dose level of 25 mg. daily.

(3) The growth hormone was a water soluble product which, although not chemically pure, was reported to be free of all other biologically active components of the pituitary. Daily doses of 0·5 mg. were administered in water solution by subcutaneous injection.

The beef anterior pituitary preparation was administered to female, male, and ovariectomized female rats (Series F5–C, F6, F8, F11 and 4F–3). In addition, female castrates and males were treated with both the anterior pituitary and diethylstilbestrol (5 $\mu$g. daily or 10 $\mu$g. every second day), (Series F6 and F9–B). The sheep pituitary extract and the growth hormone were tested in single experiments with female rats (Series F9–A and F–12). Representative results are given in Table VI

Both beef anterior pituitary and sheep pituitary preparations stimulated the growth of the fibroadenomata. The former was active in males and castrated

TABLE VI.—*Effect of Various Pituitary Preparations on the Growth of Transplanted Fibroadenomata in Rats.*

| Series. | Treatment.* | Sex. | Number of rats. | Number of takes. | Mean latent period (days). | Mean growth period (days). |
|---|---|---|---|---|---|---|
| F6 | A.P.S.,† limited | F | 8 | 8 | 22± 1·1 | 21± 1·0‡ |
| | Controls, intact | F | 10 | 9 | 43±15·4 | 35± 3·2 |
| | Ovariectomy + A.P.S., limited | F | 8 | 8 | 24± 1·0 | 23± 1·9‡ |
| | Ovariectomy + (A.P.S. and Stilbestrol), limited | F | 8 | 8 | 21± 1·2‖ | 27± 1·4‡ |
| | Ovariectomized controls | F | 8 | 7 | 29± 2·9 | 37± 1·6 |
| F11 | A.P.S., continuous | F | 10 | 5 | 46± 2·2 | 43± 7·1 |
| | Controls, intact | F | 10 | 7 | 61± 6·2 | 56±21·9 |
| | Ovariectomy + A.P.S., continuous | F | 10 | 5 | 56± 5·7 | 59±15·1 |
| | Ovariectomized controls | F | 10 | 0 | — | — |
| F8 | A.P.S., continuous | M | 10 | 9 | 38± 2·2 | 26± 2·0‖ |
| | Controls | M | 10 | 10 | 45± 3·4 | 32± 1·6 |
| F9–B | A.P.S., continuous | M | 10 | 4 | 80±14·6 | 32± 3·2 |
| | A.P.S. + stilbestrol, continuous | M | 10 | 8 | 69± 4·6 | 78±13·6 |
| | Controls | M | 10 | 0 | — | — |
| F9–A | Sheep pituitary extract, continuous | F | 10 | 8 | 34± 1·7 | 29± 0·8‖ |
| | Controls | F | 10 | 7 | 41± 3·6 | 35± 2·0 |
| F12 | Growth Hormone, continuous | F | 10 | 8 | 48± 5·1 | 43± 9·7 |
| | Controls | F | 10 | 7 | 42± 6·1 | 39± 3·9 |

* Treatment, for dose levels see description in text.
† A.P.S., beef anterior pituitary suspension.
‡ $P < 0·01$, treated versus controls.
‖ $P < 0·05$, treated versus controls.

females as well as in intact females. During the course of some of the experiments using the beef anterior pituitary mixture, penicillin was administered for a short period to allay infection induced by the injected material. This treatment proved to be satisfactory and did not appear to influence the tumour growth. The action of beef anterior pituitary was not evident in all experiments with intact females (Series F9–B and F11). In these cases, however, the individual variation was extreme in both control and treated animals, a condition which may have masked any existing response. Stilbestrol did not appear to augment the anterior pituitary stimulation of fibroadenomata in castrated females or in males.

In the single experiment conducted, there was no evidence of a response to pure growth hormone.

There was no change in the morphology of the benign tumours or the incidence of fibrosarcomata with any of the treatment regimes given.

## DISCUSSION.

Although the fibroadenoma is more successfully propagated in the female host, œstrogen administered in small doses to intact rats caused no change in tumour growth or morphology, except in one early experiment (F3). This one exception may be attributed to chance variation or can be interpreted as evidence of a greater

responsiveness in early tumour generations. This possibility is supported by the findings of Heiman and Mohs, which were reviewed earlier, but is not upheld by the later work using third generation tumours of Lines 3F and 4F.

In male rats, however, small amounts of exogenous estrogen produced tumour growth and morphology comparable to that of female controls. This response supplies further proof for the need of minimal estrogen levels for maximum growth of the mammary fibroadenoma in rats. Depression of the male sex hormone may also be a factor involved, particularly with regard to the increased epithelial development of tumours in estrogen treated males. The inhibition of tumour epithelium by testosterone was postulated in the preceding paper (Millar and Noble, 1954), when it was observed that tumours were more fibrous in male rats than in female controls, but were unchanged morphologically in castrated females. Experiments with administered androgens tend to support this contention. Mohs (1941) reported that prolonged injections of small doses of testosterone brought about a progressive loss of tumour epithelium but no change in growth rate. Using a higher dose level and shorter treatment period, in this laboratory the tumours tended to be more fibrous and showed only slight growth inhibition. Heiman (1940*a* and 1943) reported a similar but more dramatic response to the action of testosterone on both growth rate and epithelial development. Dose levels, not clearly defined, were probably intermediate to those used by Mohs (1941) and those used in this laboratory. High susceptibility to change seems typical of the tumours studied by this worker. This may be a characteristic of the strain of rat used, for the Sprague-Dawley strain employed by us was also used by Mohs.

The negative response of fibroadenomata to administered progesterone reported herein, as opposed to the marked inhibition observed by Heiman (1940*a* and 1943) offers another example of varied susceptibility. Although in our experiments, progesterone treatment was for the 4-week period only, the relatively undramatic effect of large doses of testosterone in intact females makes a marked response to progesterone rather difficult to envision even if the treatment period had been prolonged.

Inhibition of the growth of transplanted mammary fibroadenomata produced by high doses of estrogen has not been reported by other workers. To the authors' knowledge Emge (1939) is the only worker to use similar high levels of estrogens in studies on mammary fibroadenomata. His experiments were designed to study the effect of such treatment on the incidence of carcinoma in these benign tumours. Marked epithelial hyperplasia, but no evidence of carcinomatous transformation was observed. It is assumed that the treatment occurred after tumour growth-initiation. No reference is made to alterations in tumour growth rate. A systematic study of changes in tumour morphology during treatment has not been carried out in this laboratory, but examination of tumours from a few rats which died during treatment periods, gave no indication of the pronounced epithelial response observed by Emge (1939). This again demonstrates the limited capacity of the epithelium of the tumours observed by us to respond to environmental changes without the physiological range.

Considerable speculation could be devoted to the mode of action of stilbestrol on the growth of fibroadenomata. The most obvious possibility, i.e., that of a nonspecific reflection of body growth depression, has been ruled out by paired feeding experiments. The fibroadenoma may be responding like normal immature

mammary tissue, for it has been shown that mammary gland development in young rats is depressed by large doses of estrogen (Astwood, Geschickter and Rausch, 1937). It is well known that excessive estrogen will inhibit the growth of some malignant neoplasms in rats and mice (Eisen, 1941 ; Nathanson and Salter, 1939) and in humans such action is well exemplified by the use of estrogen therapy for breast cancer. The variability of response in humans (Nathanson and Kelley, 1952) brings to mind the all or none response obtained with stilbestrol administration to rats bearing growing fibroadenomata. It is difficult to assess to what extent the benign fibroadenoma can be compared with malignant growths or to normal tissue with regard to the mechanism of response to estrogen excesses. Nevertheless, the hormonal imbalance created, with the resulting depression of anterior pituitary function, very likely plays an important part in the inhibition of the benign fibroadenoma in other types of breast tumours and as well in normal breast tissue.

From a therapeutic point of view the desirability of such a response in the fibroadenoma is questionable, since the incidence of malignant transformations was increased in the groups treated during tumour growth, this event being coincident with tumour growth inhibition. In the preceding paper (Millar and Noble, 1954) it was shown that the change to fibrosarcoma was restricted to slow growing fibroadenomata and it was proposed that the fibrosarcomatous transformation was instigated in an effort to overcome the action of some intrinsic inhibitory agent. Apparently, contradictory to this theory, was the lack of an increased incidence of fibrosarcomata in the tumours of rats injected with high doses of stilbestrol from the time of tumour implantation, where artificially prolonged latent periods of over 200 days were observed. In view of this more dramatic growth response it is difficult to understand why stilbestrol administration during tumours growth should increase the incidence of fibrosarcomata. Possibly the treatment or the resulting growth inhibition is more effective in bringing about a fibrosarcomatous transformation in established tumours which have been actively growing than in the temporarily dormant implant.

The findings reported for the action of anterior pituitary preparations on the mammary fibroadenoma are of particular interest in view of the currently increasing evidence of the part played by pituitary hormones in carcinogenesis (Moon, Simpson, Li and Evans, 1950 ; Moon, Simpson and Evans, 1952 ; Noble and Walters, 1954).

The identity of the pituitary component or components which stimulate the growth of the fibroadenoma is not known. Since both the beef and sheep pituitary preparations used were crude products, the response was probably a result of the integrated action of several pituitary hormones. However, if a single hormone was responsible for the increased tumour growth rates observed, prolactin would seem to be the most likely possibility. Gonadotrophic activity was low in the beef pituitary preparation, as was growth hormone activity in the sheep pituitary extract. Negative results with purified growth hormone further justifies its elimination. Prolactin activity was high in both crude preparations. Adrenocorticotrophin and thyrotrophin were probably present and cannot be eliminated as possible active factors, but one is inclined to favour prolactin in view of its important role in mammary tissue development and lactation (Folley, 1952).

The lack of response to purified growth hormone confirms the findings of Heiman and Krehbiel (1936). However, no synergism was noted between estrogen

and the beef anterior pituitary preparation, while these workers reported that estrogen plus gonadotrophin or growth hormone stimulated tumour growth although none of the individual products were active in intact rats.

In conclusion, a review of the findings reported herein and elsewhere leads to the realization that in the mammary fibroadenoma one is dealing with a tissue possessing both normal and neoplastic properties. Being of mammary origin these tumours frequently respond to hormones involved in mammary gland development, but the type and degree of response often differs widely from that expected of normal tissue. Thus, testosterone which stimulates alveolar development in mammary tissue, depresses the epithelium of transplanted fibroadenomata. Alternately, estrogen at physiological levels which normally stimulate epithelial development alone promotes the growth of these predominantly connective tissue tumours. Estrogen excesses may or may not increase glandular tissue in these tumours, the variation between tumour lines and strains of rats being marked in this respect. Finally, cortisone which causes the breakdown of some collagen connective tissues, is ineffective in altering the growth characteristics of the mammary fibroadenoma.

## SUMMARY.

1. The administration of small doses of diethylstilbestrol to female rats did not alter the growth characteristics or morphology of transplanted mammary fibroadenomata. However, tumour growth rate and epithelial development in similarly treated male rats was increased to equal that of female controls.

2. High levels of diethylstilbestrol (100–200 $\mu$g. daily) prevented tumour growth initiation when administered from the time of tumour implantation. If treatment was commenced after tumour growth initiation, the response was less uniform, but if any, was one of growth retardation or arrest. The incidence of fibrosarcomata was increased in the latter group and this event was restricted to the fibroadenomata showing slowed or arrested growth.

3. Testosterone proprionate produced slight tumour growth inhibition and an indication of depressed tumour epithelial development. Experimental findings were inconclusive but the results of others support the trends observed.

4. Progesterone and cortisone in the amounts and for the duration administered did not alter the growth rate or morphology of transplanted fibroadenomata in female rats.

5. Crude beef and sheep pituitary preparations stimulated tumour growth in intact female rats. The former was also active in males and castrated females. The simultaneous administration of small doses of estrogen did not augment the action of the beef pituitary preparation. Biologically pure growth hormone did not alter the growth or morphology of the transplanted fibroadenomata in female rats.

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## REFERENCES.

ASTWOOD, E. B., GESCHICKTER, C. F., AND RAUSCH, E. O.—(1937) *Amer. J. Anat.*, **61**, 373.

EISEN, M. J.—(1941) *Cancer Res.*, **1**, 457.

EMGE, L. A.—(1939) *Surg., Gynec. and Obstet.*, **68**, 472.
*Idem* AND MURPHY, K. M.—(1938) *Proc. Soc. exp. Biol., N.Y.*, **37**, 620.
*Iidem* AND SCHILLING, W.—(1938) *Ibid.*, **38**, 21.
FOLLEY, S. J.—(1952) *Recent Progr. Hormone Res.*, **7**, 107.
HEIMAN, J.—(1940*a*) *Amer. J. Cancer*, **39**, 178.—(1940*b*) *Ibid.*, **40**, 343.—(1943) *Cancer Res.*, **3**, 65.
*Idem* AND KREHBIEL, F.—(1936) *Amer. J. Cancer*, **27**, 450.
MILLAR, M. J., AND NOBLE, R. L.—(1952) *Cancer Res.*, **12**, 282.—(1954) *Brit. J. Cancer*, **8**, 485.
MOHS, F. E.—(1940) *Amer. J. Cancer*, **38**, 212.—(1941) *Cancer Res.*, **1**, 151.
MOON, H. D., SIMPSON, M. E., AND EVANS, H. M.—(1952) *Science*, **116**, 331.
*Idem*, SIMPSON, M. E., LI, C. H., AND EVANS, H. M.—(1950) *Cancer Res.*, **10**, 297.
MURPHY, K. M., SCHILLING, W., AND EMGE, L. A.—(1938) *Proc. Soc. exp. Biol., N.Y.*, **39**, 298.
NATHANSON, I. T., AND KELLEY, R. M.—(1952) *New Engl. J. Med.*, **246**, 135.
*Idem* AND SALTER, W. T.—(1939) *Arch. Path.*, **27**, 828.
NOBLE, R. L., AND WALTERS, J. H.—(1954) *Proc. Amer. Ass. Cancer Res*, **1**, 35.

# THE GROWTH CHARACTERISTICS AND RESPONSE TO HORMONES OF TRANSPLANTED FIBROSARCOMA ARISING FROM MAMMARY FIBROADENOMA IN THE RAT.

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THE growth characteristics and morphology of transplanted mammary fibroadenomata and the effect of endogenous and exogenous hormones upon them have been presented in the two preceding papers (Millar and Noble, 1954*a* and 1954*b*). A transformation to fibrosarcoma occurred fairly frequently in such benign tumours and in many cases it was possible to detect fibrosarcomatous tissue within a fibroadenoma by macroscopic examination alone. By transplanting this tumour tissue many sub-lines of fibrosarcomata have been developed from the single line of fibroadenomata studied. The growth characteristics and pathology of these sub-lines are described briefly herein and compared with the fibroadenomata from which they originated.

There is considerable evidence in the literature to indicate that the fibrosarcomatous transformation constitutes a permanent modification of the growth characteristics of the original fibroadenoma. Increased takes (Heiman, 1934; Selbie, 1941) and growth-rate (Emge, 1938) have been noted when transplanted fibrosarcomata of fibroadenomatous origin were compared with the fibroadenoma. These tumours are reported to grow equally well in male and female rats (Oberling, Guerin and Guerin, 1935; Selbie, 1941) and in rats of different strains (Heiman 1934), characteristics not common to fibroadenomata. Heiman (1934) also found that the amount of X-radiation required to kill fibrosarcoma and fibroadenoma cells was 3500 r and 5500 r respectively. In order to produce further evidence of this alteration in response to environmental conditions, experiments were undertaken to determine the effect on transplanted fibrosarcomata of hormonal preparations known to influence the growth of the benign fibroadenoma. These were diethylstilbestrol at high dose levels and an anterior pituitary preparation. The former depressed while the latter enhanced fibroadenoma growth (Millar and Noble, 1954*b*).

## METHODS.

Fibrosarcoma lines developed from fibroadenoma were identified by the letters *FM.*— : a number before these letters distinguishing the individual lines and a number following, the transplant generation. At least one tumour of each transplant generation was examined microscopically.

Young adult female rats of the Sprague-Dawley strain were used in these studies. Details of transplantation technique (using single axillary implants) and the method for assessing the effects of different treatments within transplant

series is given in the first of the two preceding papers (Millar and Noble, 1954*a*). For convenience a brief review of the latter is given below. After growth initiation, the tumours were measured once or twice weekly (depending on the growth-rate) till the death of the animal. Tumour size (in square inches) was plotted against time and from such growth curves figures were obtained for " latent " and " growth " periods. These were defined respectively as the time from the implantation date till the tumour was 0·4 square inches in area and the time for the tumour to grow from 0·4 to 3·0 square inches. Mean tumour latent and growth periods for treated and control groups were compared by the " t " test of Fisher, using a probability of 0·05 as the level of significance. All means will be presented with their standard errors.

Three separate experiments using different FM tumour lines were conducted to determine the effect of estrogen at high dose levels and a beef anterior pituitary suspension on the growth of the fibrosarcomata. Diethylstilbestrol was given orally as a constituent of the drinking water (0·5 mg. per cent) or by subcutaneous injection in sesame oil solution. Dose levels were approximately 50 μg. daily by the former and 200 μg. every second day by the latter mode of administration. As an added control in one experiment, a group of rats was subjected to dietary restriction sufficient to produce weight loss comparable to that observed in the estrogen injected rats. Preparation of the beef anterior pituitary saline suspension is outlined in a previous paper (Millar and Noble, 1954*b*). It was administered every second day by subcutaneous injection in an amount equivalent to 200 mg. of anterior pituitary substance (1 ml.). All treatment began at the time of tumour implantation and was continued throughout the duration of the experiment.

## RESULTS.

### (1) *Pathology.*

The first paper of this series (Millar and Noble, 1954*a*) gave a detailed description of the macroscopic and microscopic pictures of fibroadenomata which have undergone a fibrosarcomatous transformation. Variation in the degree of cellularily, anaplasia and mitotic activity led to the classification of this tissue as suspicious-low, low, moderate and high grade fibrosarcoma. Bony and cartilagenous metaplasia was observed in some tumours and has become quite characteristic of one FM tumour line after the second generation. Photomicrographs of a high grade fibrosarcoma and the metaplastic change were shown in the earlier paper (Millar and Noble, 1954*a*, Fig. 6 and Fig. 7).

Forty-two fibroadenomata suspected of having undergone a change to fibrosarcoma were transplanted. Of these, six grew as unchanged fibroadenomata, two were identified as fibroma, while the remainder fell into the above grades of fibrosarcomata. Although the bulk of the FM lines were classified initially as suspicious low-grade or low-grade fibrosarcomata, these tumours tended to change to the more cellular, anaplastic forms in the course of transplantation. In only one instance was there a reversion to a lower grade (37 FM to 37 FM1—moderate to low-grade fibrosarcoma).

### (2) *Growth characteristics of FM tumour lines.*

The growth energy of FM tumour lines varied considerably. The percentage takes ranged between zero and 100 ; the majority being 50 to 75 per cent in small

groups of four or five rats. Latent periods for first generation tumours ranged from 7 to 61 days. Growth was usually fairly rapid, but as the tumours were measured only when experiments such as those to be reported were being conducted, quantitative data is not complete for this aspect. The mean "growth periods" given in Table I are representative of most of the tumours observed, although considerably slower growth than these figures indicate was not uncommon. Metastases have not been observed.

Correlation between the pathological grade of fibrosarcoma and the growth characteristics was by no means clear cut. With reference to the latent periods given above, only the lines originating from high-grade fibrosarcomata gave any indication of being a distinct group. The range for these tumours was 8 to 20 days, while the lower grades varied within the limits given, i.e., 7 to 61 days. In general it can be said only that the chances of obtaining rapidly growing, easily transplantable tumours, from tissue classified as moderate or high-grade fibrosarcoma exceed those from the two lower grades.

The pathological change from fibroadenoma to fibrosarcoma was accompanied by alterations in growth characteristics of the tumour and the response of the host to the tumour mass. Although there was considerable overlap in the range of latent and growth periods for fibroadenomata and fibrosarcomata, as a group of the FM tumours were more readily propagated and faster growing. The effect of the fibrosarcomata on the general health of the host varied considerably for individual tumours. Slow growing tumours were usually well tolerated, while the more rapidly growing ones were more deleterious to the health of the tumour-bearer. However, in no instance has an FM tumour reached a size of over 150 g. (5·0 to 7·0 square inches) without causing weight loss and cachexia. Death has occurred in some animals before the tumour has reached 3·0 square inches in size and could be attributed to no other obvious pathology. The depletion during the growth period of several groups may be noted in Table I. On the other hand, fibroadenomata of size equal to or exceeding the body-weight of the host do not appear to harm the tumour-bearer except through the physical disability resulting from the enormous tumour mass.

(3) *The effect of estrogen at high dose levels and an anterior pituitary preparation on the growth of FM tumours.*

The tumour lines used in these experiments were as follows:

15 FM2—: high-grade fibrosarcoma
35 FM5— : moderate-grade fibrosarcoma (35 FM4—: low-grade fibrosarcoma)
42 FM2— : high-grade fibrosarcoma.

The results for the experimental groups of each tumour line are given in Table I.

The mean latent period and the mean growth period of 15 FM and 35 FM lines respectively were significantly longer in diethylstilbestrol treated rats. A similar trend was noted in the 42 FM line, but the differences were not significant. Dietary restriction caused no retardation in tumour-growth in this experiment. The anterior pituitary suspension did not alter the growth trend of any of the three tumour lines studied.

TABLE I.—*The Effect of Estrogen at High Dose Levels and an Anterior Pituitary Suspension on the Growth of FM Tumours.*

| Tumour line. | Treatment. | No. of rats. | No. of takes. | Mean latent period (days). | Mean growth period (days). |
|---|---|---|---|---|---|
| 15 FM | Diethylstilbestrol* | 8 | 8 | 13±1·4‖ | 22±3·1 (5 rats) |
| | Anterior pituitary suspension | 8 | 8 | 9±0·3 | 18±2·5 |
| | Controls | 8 | 5 | 9±0·4 | 18±1·9 |
| 35 FM | Diethylstilbestrol† | 8 | 8 | 26±1·0 | 22±1·5‖ (6 rats) |
| | Anterior pituitary suspension | 8 | 6 | 25±0·6 | 13±1·3 (4 rats) |
| | Controls | 8 | 6 | 27±1·4 | 15±1·6 (5 rats) |
| 42 FM | Diethylstilbestrol† | 8 | 8 | 17±1·1 | 23±1·6 (7 rats) |
| | Anterior pituitary suspension | 8 | 8 | 15±1·5 | 18±1·1 |
| | Dietary restriction‡ | 8 | 8 | 13±0·4 | 19±1·9 |
| | Controls | 8 | 8 | 14±1·4 | 21±1·8 (7 rats) |

* Diethylstilbestrol given orally in drinking water—approximately 50 μg. daily.
† Diethylstilbestrol injected subcutaneously—200 μg. every second day.
‡ Dietary restriction—sufficient to produce weight loss, comparable to that observed in diethylstilbestrol injected rats.
‖ $P < 0{\cdot}05$—Treated versus controls.

## DISCUSSION.

The results presented are in accordance with those reported by others. The sub-lines of fibrosarcomata arising from a single fibroadenoma line produced in general more rapidly growing, independent tumours than the fibroadenomata. The body weight loss and early death of fibrosarcoma-bearing rats was not observed for rats bearing fibroadenomata of similar or much larger size. Similarly, Emge (1938) reported that such fibrosarcomata caused the death of the host in 30 to 40 days. Metabolic differences have also been noted. Begg, Dickinson and Millar (1953) observed that the reduction in liver catalase activity was significantly greater in rats bearing transplanted fibrosarcomata or fibroadenomata of which a portion had become sarcomatous than in rats bearing totally benign fibroadenomata.

The histological appearance and the growth properties point to the classification of these tumours as malignant growths. Metastases have not been observed in FM tumour-bearing rats, but Emge (1938) noted that these fibrosarcomata recurred rapidly after surgical removal, while Heiman (1934) observed local invasion into the chest wall of one such tumour. Increased autonomy as shown by diminished or negative response to hormonal factors known to influence the fibroadenoma offers further evidence of the true malignancy of these growths. Diethylstilbestrol administered at similar dose levels prevented the initiation of growth in fibroadenoma implants, or arrested or markedly slowed the growth of growing tumours (Millar and Noble 1954*b*). The comparatively slight, but usually significant growth retardation shown by FM tumours was similar to that observed with estrogen administration for other malignant growths, including sarcomata (Nathanson and Salter, 1939; Eisen, 1941). Fibroadenoma-stimulating hormones present in beef anterior pituitaries did not enhance the growth of FM tumours. This confirms the alternate findings of Emge and Murphy (1936)

that growth of similar tumours in hypophysectomised rats was only slightly depressed, if at all.

The existence and propagation of benign and malignant growths of common origin offers a tool for cancer research which may be of considerable value. The use of the benign tumour-bearer as a more representative control for the study of malignant tumour-host relationships has already been reported for liver-catalase studies by Begg, Dickinson and Millar (1953). It is possible too that the more gradual development of malignancy from normal tissue through the benign neoplasm, would reveal in metabolic studies more subtle changes than can be detected in comparing only normal and malignant tissue of common origin.

SUMMARY.

1. The pathology and growth characteristics of tumour lines originating from fibrosarcomatous tissue occurring in transplanted mammary fibroadenomata are described.

2. In comparing these tumours with the benign growths of their origin, it was noted that the fibrosarcomata were usually faster growing and more easily propagated than the fibroadenomata. In addition they exerted a deleterious effect upon the health of the host, causing weight loss and death. No comparable response was observed in rats bearing fibroadenomata.

3. Diethylstilbestrol at high dose levels caused slight but usually significant retardation of tumour growth, a response similar but not comparable in degree to that observed with the fibroadenoma.

4. A crude beef anterior pituitary suspension known to enhance the growth of fibroadenomata did not alter the growth energy of fibrosarcomata.

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Miss Bette Byrns rendered valuable technical assistance in these experiments.

## REFERENCES.

BEGG, R. W., DICKINSON, T. E., AND MILLAR, J.—(1953) *Canad. J. med. Sci.*, **31**, 315.
EISEN, M. J.—(1941) *Cancer Res.*, **1**, 457.
EMGE, L. A.—(1938) *Arch. Path.*, **26**, 429.
*Idem* AND MURPHY, K. M.—(1936) *Amer. J. Obstet. Gynec.*, **32**, 593.
HEIMAN, J.—(1934) *Amer. J. Cancer*, **22**, 497.
MILLAR, M. J., AND NOBLE, R. L.—(1954*a*) *Brit. J. Cancer*, **8**, 485.—(1954*b*) *Ibid.*, **8**, 495.
NATHANSON, I. T., AND SALTER, W. T.—(1939) *Arch. Path.*, **27**, 828.
OBERLING, C., GUERIN, M., AND GUERIN, P.—(1935) *Bull. Ass. franc. Cancer*, **24**, 232.
SELBIE, F. R.—(1941) *Brit. J. exp. Path.*, **22**, 156.

# THE EFFECT OF "SYNKAVIT" ADMINISTRATION COMBINED WITH IRRADIATION UPON RABBITS AND RATS.

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AMONGST the attempts to improve the results in radiotherapy of some types of cancer, especially those in which results are unsatisfactory, by the ancillary use of chemical agents designed to act as radiosensitizers, the work of Mitchell (1948) has been most consistently and thoroughly pursued. After many clinical trials Mitchell (1950, 1952, 1953) has shown that the administration of tetrasodium 2-methyl-1 : 4-napthohydroquinone diphosphate (synthetic vitamin K derivative, Synkavit, Roche) to patients with advanced malignant disease improves significantly the survival results of treatment. Laboratory tests with tissue cultures and animal experiments, with a large series of chemical compounds (Mitchell and Simon-Reuss, 1952*a*, 1952*b*) have shown the superiority of this temporarily solubilised quinol.

Clinical trials with the compound were made in this department, and intramuscular and later intravenous injections of 100 mg. of Synkavit were given daily to patients as a coadjuvant to radiation treatment for advanced carcinomas. Some 3½ years ago a striking improvement in the general condition of the majority of 100 patients undergoing radiotherapy for advanced cancer, to whom Synkavit injections were given had been observed. This might be attributed to an increased tolerance of radiation, and this possibility led to a series of animal experiments in which a comparison was made between the lethal effects of X-rays in animals receiving Synkavit prior to and after irradiation with control animals which received X-rays and a series of injections of saline, and animals which received X-rays and no injections at all.

The work here described is concerned with the animal experiments only as the clinical material available does not allow data regarding the effectiveness of Synkavit as a coadjuvant of radiotherapy as yet to be presented.

## MATERIALS AND METHODS.

Sixty-five rabbits and 75 rats were used in these experiments.

The rabbits were Old English, farm bred, and of a non-homogeneous strain. Attempts were always made to use in sets of experiments to be compared animals of similar age and body weight.

The rats were of the "Birmingham Strain," obtained by the courtesy of Dr. A. M. Mandl, Department of Anatomy, Birmingham University. Their weights at commencement of experiment were 200 $\pm$ 10 g. and were taken daily or every other day. Food and water were supplied *ad libitum* otherwise the general maintenance and handling of the animals was standardised.

Thirty-five rabbits and 37 rats were given subcutaneous injections of Synkavit, the amounts given to rabbits ranging from 10–50 mg. daily, but constantly 10 mg. in the case of rats. The remainder served as controls. The drug was given in some cases for a few days before the delivery of the X-ray dose and continued for a time varying from 10–17 days. The dose of X-rays given in the case of rabbits ranged from 600–1500 r. There were three groups of 26, 24 and 25 rats which received respectively 480 r, 580 r and 650 r.

All the rabbits and all the rats were exposed to a beam of radiation covering the whole body. The physical factors were 180 kV 10mA. 50 cm. F.S.D. with a H.V.L. varying according to the filtration, which was either the inherent tube shield filtration alone or 0·5 mm. Cu. (H.V.L. 0·85 mm. Cu.). The irradiation was carried out with the tube head and the applicator inverted, with the box containing the animals standing on top of the applicator end, the incident beam falling on the ventral surface of the animals. The rabbits were not anaesthetised but kept still by padding the box and its partitions. Autopsy was performed in almost all animals dying as a result of irradiation, and those which were killed after they had survived a period of 30–50 days. Sections were taken of the lung, spleen, adrenals, liver and testes in almost all cases.

## RESULTS.

### *Rabbits.*

The difference in survival rates between the Synkavit plus X-rays' series of rabbits with the series of rabbits which received X-rays alone was not significant when the assessment was made on the whole series of 35 rabbits which received Synkavit injections. Fourteen out of 35 rabbits survived the 30 days' period, the remaining 21 survived on an average 8 days after a dose of 800–1500 r had been given in conjunction with Synkavit. The amount of the latter varied according to the number of injections given, which in many animals was cut short by death.

In a control series 5 out of 12 survived similar doses of X-rays without Synkavit, the remaining 7 dying within an average of 7·5 days. The weight curve of these animals differed in the two series considerably. In the Synkavit group (Fig. 1) the majority maintained their weight fairly well throughout the observation period or gained ground slightly. This was particularly so in the case of the rabbits which received Synkavit injections for a few days prior to exposure to X-rays. In the series of rabbits exposed to X-rays without Synkavit (Fig. 2) there was a consistent drop in the weight curve, with the exception of one surviving rabbit which gained some 10 per cent in weight over a period of 30 days. The Synkavit rabbits were on the whole more lively and taking food more liberally than the animals without Synkavit.

### *Rats.*

The 75 rats used in these experiments were dealt with in three separate series.

*Group I.*—Thirteen rats received 10 mg. of Synkavit injections daily (7 rats for 4 days and 6 rats for 5 days) prior to exposure to a dose of 580 r and subsequently daily until their death, and a group of 13 rats received a similar dose of X-rays to the whole body without any injections. In the Synkavit group there was 1 survivor and in the non-Synkavit series there were no survivors at 30 days.

While in the Synkavit series 3 deaths occurred within 15 days there were no deaths before 21 days in the X-rayed group not receiving the compound (Fig. 3).

*Group II*.—Twenty-four rats were exposed to a dose of 650 r to the whole body. Of these, 11 were given injections of 10 mg. of Synkavit daily, and 13 were given daily injections of 1 ml. of sterile normal solution of saline. There were no 30-day survivals in the whole series (Fig. 4). Of the 11 Synkavit rats, 7 died at 4 days, 2 at 6 days, 1 at 7 days and 1 at 9 days after irradition. In the saline group the deaths occurred : 1 rat at 3 days, 5 at 4 days, 2 at 6 days, 2 at 7 days, 1 at 8 days, 1 at 9 days and 1 at 15 days.

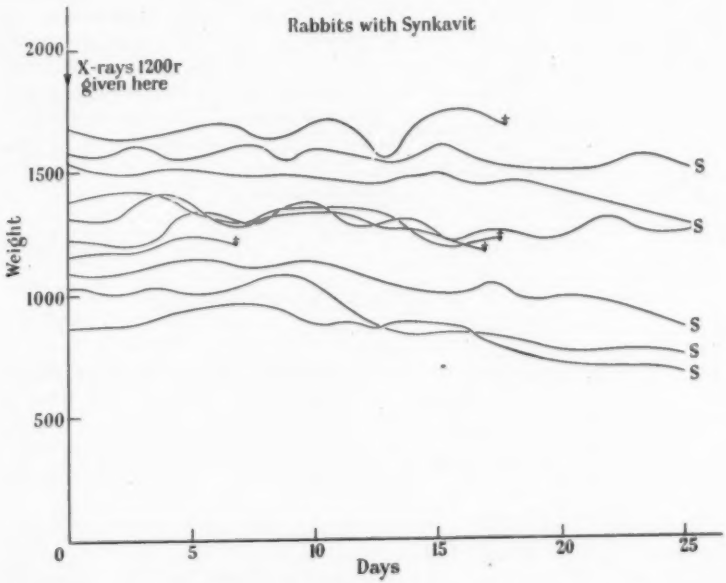


FIG. 1.—Body weight and survival (S) curves of irradiated rabbits which received Synkavit injections.

*Group III* (Fig. 5).—A group of 25 rats were exposed to a dose of 480 r to the whole body. Of these 9 received seven and 4 received five injections, 10 mg. daily, of Synkavit prior to exposure to X-rays and eight to eleven daily injections after exposure. Eight rats received six pre-X-ray treatment and 10 post-treatment injections of 1 c.c. of sterile normal solution of saline and 4 received no injections whatsoever. In the group of rats which received saline injections there were no deaths at 31 days after irradition. In the group of rats which received Synkavit injections there was only 1 survival at 31 days, the remainder dying within 12–16 days. The 4 control (X-ray only) rats survived 31 days. As regards the weight curve in the 580 r series, all the Synkavit rats showed a loss which varied from 15 to 30 per cent, of their body weight, while in the non-Synkavit series there was also a consistent loss of weight though perhaps to a lesser extent. In the 650 r series the death of the animals occurred so rapidly after the irradiation

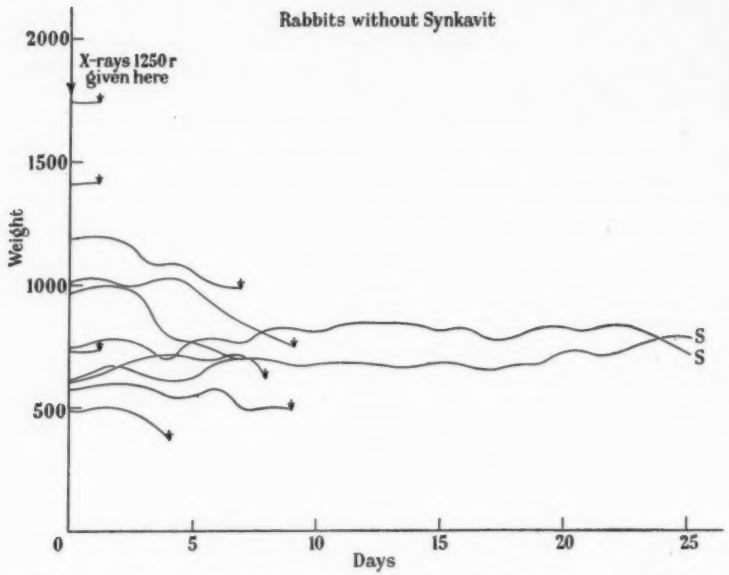


FIG. 2.—Body weight and survival curves of irradiated rabbits which received no injections.

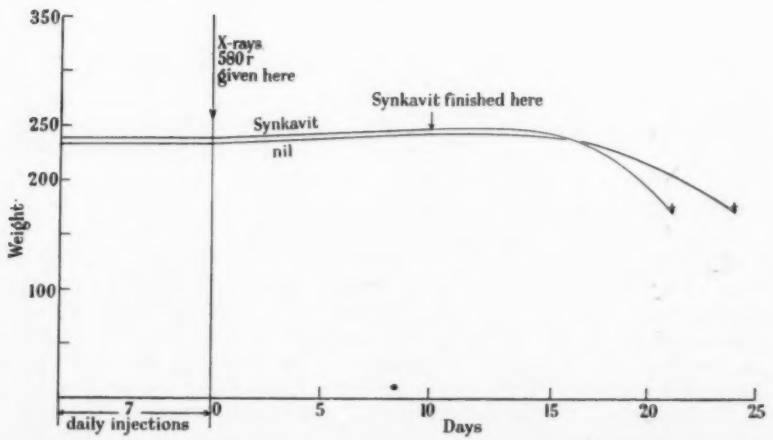


FIG. 3.—Survival times of 26 rats of which 13 received Synkavit injections prior to and after irradiation (580 r) and 13 were irradiated but did not receive any injections.

that few weight data are available. The same applies to the 480 r Synkavit rats while in the saline and control 480 r rats a consistent drop in weight was noted.

*Microscopical.*

The spleen and adrenals of both rats and rabbits were examined in these experiments. The adrenal has been comparatively little studied, and yet is considered

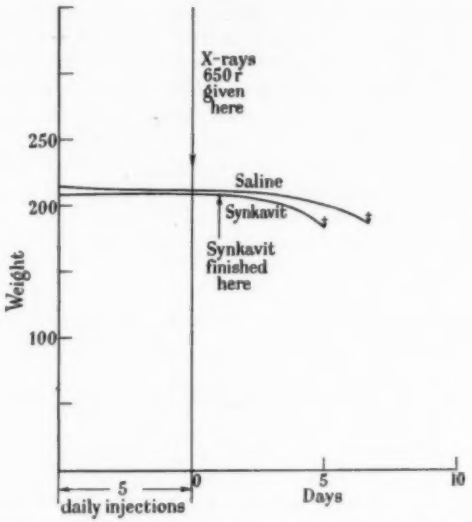


FIG. 4.—Survival times of 24 rats (12 with Synkavit and 12 with saline injections) given a dose of 650 r.

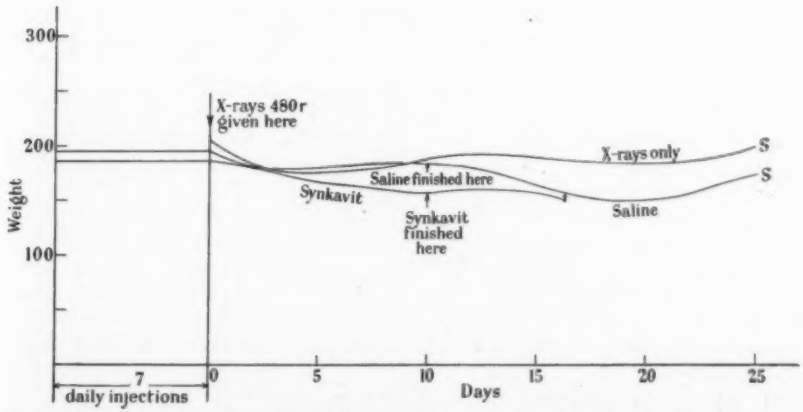


FIG. 5.—Survival times of 25 rats (13 with Synkavit, 8 with normal saline injections and 4 without any injections) exposed to 480 r.

in the recent literature to play some part in the syndrome of irradiation sickness (Porter, 1952) and in the repair of tissue damage following irradiation (Craver, 1948). The importance of adrenal hormones in maintaining many vital functions also suggest a potential connection between this organ and the widespread changes present after irradiation.

*Spleen.*

The changes in the spleen followed the same pattern in both species and were of the type previously described in the mouse by Jacobson *et al.* (1950). The lymphoid follicles disappeared rapidly in the first 3 weeks. Those surviving for longer periods however often presented a relatively normal appearance in the lymphoid nodules, due to regeneration following, perhaps, less initial damage, though this can only be surmised. In many of the animals dying in the earlier period there were further changes of an atrophic nature, in some cases little except blood vessels and fibrous tissues being seen. Iron containing pigment was often prominent in these specimens. There was in general no difference between animals given Synkavit and the controls dying after the same period.

*Adrenal.*

The histopathology of the adrenal following direct irradiation has received surprisingly little attention, especially from modern workers. As Rhoades remarked in 1948 the material available is slight and mostly contradictory. The chief papers are those of Frey (1928), Engelstad and Torgersen (1937) and Warren (1943). The first concerns the guinea-pig and under the conditions of the experiment the author concluded that there was no direct effect of the radiation on the cortex. Engelstad and Torgersen (1937), working with rabbits, decided on the other hand that with high dosage there was a marked effect leading to degeneration of the cortex over a period starting a few days after the irradiation, and, in surviving rabbits, progressing even for 2 or 3 months. They concluded that the radiosensitivity of the cortical tissue approximates to that of the skin. The medulla showed little change. These observations were confirmed by Warren (1943) who emphasized that it is difficult to distinguish minimal changes from those of secondary radiation effects. There appears to be no previous study of the rat adrenal.

*Rabbit.*—In general the findings in these species are similar to those of Engelstad and Torgersen (1937). The changes in the cytoplasm consist of loss of lipoid at an early stage and in some cases a marked basophilia. This latter tends to disappear but lipoid is slow to reappear in comparison with what is usually seen in secondary adrenal involvement (Nizet, Heusghem and Herve, 1949) often being small in amount at the end of 31 days. The nuclear changes affect particularly the zona fasciculata extending up to the medulla (the zona reticularis is hardly distinguishable in the rabbit). The nuclei may undergo any of the commonly seen forms of degeneration, pyknosis, fragmentation or loss of basophilia followed by dissolution. Apart from occasional initial hyperaemia there is no inflammatory reaction and one has the impression that some of the degenerated cells remain *in situ* almost indefinitely. How much function may remain in spite of the nuclear changes is an interesting point for further study.

In contrast to the constant degenerative changes in the inner zones of the cortex, the zona glomerulosa in the rabbit shows comparatively slight damage.

Both cytoplasm and nuclei appear relatively normal. No mitosis was seen in the rabbit material but in the presence of some of the relatively normal cells, its occurrence cannot be excluded on the present evidence.

*Rat.*—The normal rat adrenal differs from that of the rabbit in that the three zones are well demarcated, not only by the general cellular arrangement, but also by the nuclear character. In the zona glomerulosa the nuclei have the chromatin well dispersed with a slight concentration only on the nuclear membrane. In the fasciculata there is a tendency for it to be accumulated in small granules and this becomes very marked in the reticularis where it appears to be present mainly in masses on the nuclear membrane, giving a " clock face " appearance in many.

In the irradiated animals it was found that, unlike what had been seen in the rabbit, all zones seemed equally affected, showing cytoplasmic and nuclear changes. The cytoplasmic damage was similar to that seen in the rabbit. The nuclear alterations varied according to the normal patterns seen in the various zones. In the reticularis pyknosis was the rule ; in the fasciculata, either pyknosis or a loss of basophilia and " fading "; in the reticularis a breaking up of the nucleus, the stroma disappearing and leaving the chromatin particles scattered.

In animals dying in the first few days after irradiation, hyperaemia in the zona reticularis was common. As in the rabbit no evidence of an inflammatory reaction was seen at any time. Mitosis was in general absent but a few mitotic figures were seen in 2 rats dying on the 29th and 31st days. They were all in the outer part of the zona fasciculata.

In neither species was there any unequivocal evidence of the effect of Synkavit. In the rat, however, the general impression was one of greater damage in those receiving the compound than in the controls.

## DISCUSSION.

(1) In rabbits the administration of Synkavit prior to and after irradiation has shown slight increase in tolerance of irradiation, and although the difference in the mortality rate of rabbits and rats is not significant, the weight curve and the well-being of the animals suggested an increased tolerance of radiation in this species. In rats an opposite effect was found (Mitchell, 1951 ; Jolles, 1952). As, however, the amount of Synkavit given to rats was approximately twice as big per body weight as that given to rabbits it is quite reasonable to expect more obvious effects in the former. The findings in rabbits provide useful evidence that the amounts given to patients are not excessive, and are not likely to influence the radiosensitivity of the normal tissues which has to be avoided in clinical trials aiming at radiosensitization of tumours. The histological changes in the adrenals stained solely with haematoxylin and eosin can clearly give no more than a hint of the changes which take place in the irradiated animals. Nevertheless, enough has been found to suggest that a more detailed study of adrenal pathology and function might be fruitful in elucidating the mechanism of radiation effects. The difference in the sites of damage in the adrenals of rats and rabbits, with the comparative sparing of the zona glomerulosa in the latter, invites speculation as to whether the greater resistance of this animal to whole body radiation might be associated with a continued secretion of salt retaining hormones from this zone. Species differences in adrenocortical secretion which are genetically determined and which cannot at present be related to any known differences of adrenocortical

function have been investigated by Bush (1953). He deals however only with 3 keto-steroids in which there is no difference between rat and rabbit, although these two differ considerably from other species studied. The problem is clearly a complex one but the existence of these species' differences may in fact offer useful clues to the changes which follow irradiation. In this connection a clear distinction must be made between the direct damage to the adrenal in whole body irradiation and indirect adrenal stimulation. The salt retention found by Cori, Pucker and Goltz (1923) in sickness following localised therapeutic radiation in humans, and the transient loss of lipoid from the adrenal cortex of rabbits demonstrated by Nizet, Heusgham and Herve (1949) are almost certainly, as suggested by the latter authors, an indirect effect. The long persistence of many cells with abnormal nuclei also raises the question as to the relation of morphology and function under these circumstances.

(2) The histological findings of the effects of Synkavit are not disquieting in view of the fact that the main demonstrable effect of this compound is mitotic inhibition (Mitchell, 1949). It is perhaps not surprising that the histological appearance of tissue already showing nuclear damage and mitotic arrest from the effect of the X-rays should be little altered by the chemical. Nevertheless the suggestion of greater damage in the adrenals of rats receiving Synkavit makes it possible that this may at least have contributed to the deleterious effect of this compound in this species.

Although it is hazardous to generalise from the described experiments, the fact that patients suffering from malignant tumours who receive daily injections of Synkavit while undergoing radiotherapy stand treatment better induces one to venture an opinion that the administration of this compound in humans produces a response similar to that found in rabbits rather than that of rats. In this context, however, it has to be borne in mind that a selective concentration of the drug in tumours has been shown by Mitchell (1950) by means of ultra-violet microphotography and that the exhibition of Synkavit in normal individuals probably produces effects different from those to be expected in tumour bearing patients receiving treatment to a part of the body only.

The relatively rare occurrence of abdominal or thoracic haemorrhage as seen post mortem of Synkavit treated animals was noteworthy. The results in rats which received daily injections of 1 ml. of 0·9 per cent NaCl. solution subsequent to the exposure to X-rays are in agreement with similar findings of Ellinger and Blagg (1952) in mice.

## SUMMARY.

Experiments are described in which a comparison was made between the lethal effects of X-rays in rabbits and rats receiving Synkavit prior to and after irradiation, with control animals which received X-rays and a series of injections of saline and animals which received X-rays and no injections at all.

In rabbits the administration of Synkavit prior to and after irradiation has shown a slight increase in tolerance of irradiation, while in rats an opposite effect was found. The histological changes in the spleen and adrenals of the animals in the two series are described and the question of species differences are discussed. The findings in rabbits provide useful evidence that the amount given to patients is not excessive and is not likely to influence the radiosensitivity of

the normal tissues, which has to be avoided in clinical trials aiming at radiosensitization of tumours.

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## REFERENCES.

Bush, I. E.—(1953) *J. Endocrinol.*, **9**, 95.
Cori, K. F., Pucker, G. W., and Goltz, H.—(1923) *Amer. J. Roentgenol.*, **10**, 738.
Craver, B. N.—(1948) *Ibid.*, **59**, 404.
Ellinger, F., Blagg, J. W.—(1952) Research Report. Naval Medical Research Inst., Naval Medical Center, Bethesda, Project NM. 006.012.05.09.
Engelstadt, R. B., and Torgersen, O.—(1937) *Acta radiol., Stockh.*, **18**, 671.
Frey, H.—(1928) *Ibid.*, **9**, 23.
Jacobson, L. O., Simmons, E. L., Marks, E. K., Robson, M. J., Bethard, F. W., and Gaston, E. O.—(1950) *J. Lab. clin. Med.*, **35**, 746.
Jolles, B.—(1952) *Rep. Brit. Emp. Cancer Campgn.*, **30**, 325.
Mitchell, J. S.—(1948) *Brit. J. Cancer*, **2**, 351.—(1949) *Rep. Brit. Emp. Cancer Campgn*, **27**, 214.—(1950) *Ibid.*, **28**, 217.—(1951) *Ibid.*, **29**, 192.—(1952) *Ibid.*, **30**, 239.—(1953) *Brit. J. Cancer*, **7**, 313.
*Idem* and Simon-Reuss, I.—(1952*a*) *Ibid.*, **6**, 305.—(1952*b*) *Ibid.*, **6**, 317.
Nizet, E., Heusghem, C., and Herve, A.—(1949) *C.R. Soc. Biol., Paris*, **143**, 876.
Porter, E. C.—(1952) *Radiology*, **58**, 246.
Rhoades, R. P.—(1948) ' Histopathology of Radiation.' (Ed. W. Bloom). McGraw Hill, National Nuclear Energy Series.
Warren, S.—(1943) *Arch. Path.*, **35**, 304.

# RADIOBIOLOGY OF THE C3H MOUSE MAMMARY CARCINOMA: EFFECT OF ATTENUATION OF THE TUMOUR PRIOR TO IMPLANTATION IN $F_1$ HYBRID HOSTS.

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THE radiobiological characteristics of the C3H mammary tumour, rather than conforming to rigid quantitative values which might be attributed to intrinsic properties of the carcinoma *per se*, have been shown to change significantly as experimental variations in the host-tumour relationship were introduced. Previous work demonstrated that the $LD_{50}$ of 5700 r for treatment of the tumour *in situ* in C3H mice (Cohen and Cohen, 1953*a*) was appreciably lowered (4500 r) when homoplasts, originating primarily from viable fragments irradiated prior to implantation with sublethal doses of röntgen rays (2000–2500 r), were subsequently treated (Cohen and Cohen, 1954*b*). This increased radiosensitivity was not a permanent change in the tumour. When the growing homoplasts, arising from attenuated inocula, were sub-passaged to other hosts, the curability reverted to that of non-attenuated tumour. It was considered likely, therefore, that a subliminal resistant state had been evoked in the primary host by means of the inoculation of viable attenuated fragments, and that the resultant radiosensitivity was a quantitative measure of this resistance.

In later experiments, the radiocurability *in situ* of the C3H adenocarcinoma was determined in reciprocal $F_1$ mice, a cross between the C3H and a low-tumour CBA strain (Cohen and Cohen, 1954*a*). Here, presumably because of genetically determined differences in histocompatibility between the tumour and the hybrid hosts, the $LD_{50}$ of the tumour was significantly lower than it had been in the inbred parent strain. It was thought that the increased radiosensitivity of the tumour originating from radiation-attenuated implants in the C3H mice, and of the unattenuated tumour in the genetically alien $F_1$ hybrids, was a manifestation of a principle common to both situations, namely, that the tumour tissue was, to some extent, antigenic to its host.

In the following experiments, it was decided to determine whether the combination of attenuation and immunogenetic differences was additive, antagonistic, or neither. It was necessary first to measure the effect of irradiation *in vitro* of the C3H carcinoma prior to implantation into $F_1$ hybrids, and to estimate the radiosensitivity *in situ* of the "takes" resulting from the foregoing procedure.

## MATERIALS AND METHODS.

### *Animals.*

The mice used in this investigation were the reciprocal $F_1$ hybrid offspring of matings between our C3H/Cg high mammary tumour substrain and the low-tumour strain CBA/No, as described in a previous paper (Cohen and Cohen,

1954*a*). As before, the factor-harbouring (C3H × CBA)$F_1$ hybrid will be designated FHC, and the reciprocal (CBA × C3H)$F_1$ hybrid, presumably factor-free, as FFC.

*Tumour.*

All transplants were obtained from well established homoplasts growing in C3H parent strain hosts, the preparation of the tumour for irradiation in the plastic welled slide and its subsequent implantation by trocar being identical to that described in the first paper of the series (Cohen and Cohen, 1953*a*). The latent period after implantation varied according to the attenuation dose given, but was apparently independent of genetic or other factors, ranging from 15 to 120 days. The average latency, at any given attenuation dose, did not differ appreciably in either hybrid group from that previously observed in the parent strain host. Growth rates of the established tumour were apparently independent of the attenuation dose and were similar to those of non-attenuated tumours in the corresponding hosts. Tumours were treated *in situ* in the manner previously described when they had reached the standard size of about 1 cm. in diameter.

*Radiation Factors.*

As in all previous experiments, röntgen rays generated at 240 kVp, with no added filters, HVL 0·34 mm. Cu, FSD 25 cm., were used. The dose rate was 500 r/min. for treatment *in situ* with the 2 cm. diameter applicator, and 600 r/min. for attenuation in the plastic welled slide, using the 5 cm. diameter applicator with full back-scatter.

## EXPERIMENTAL DESIGN.

A total of 46 FHC and 57 FFC mice were used in two interdependent experiments. The first was designed to establish the median lethal dose and its standard error for the C3H carcinoma irradiated in the plastic slide immediately before implantation into hosts from each of the two hybrid groups, for comparison with the results obtained in the C3H parent strain hosts. Accordingly, three dose-levels, identical to those used in the previously reported control series, were tested: 2000 r, 2500 r, and 3000 r in each genetic subgroup. In each instance, mice from both genetic subgroups as well as C3H controls were inoculated in rotation, using the same sample of irradiated tumour tissue. For the purpose of analysis, the results in the C3H hosts were pooled with data for the same group obtained in previous experiments to form a large control series of 102 mice. The proportion of "takes" in each group was tabulated (Table I), and the $LD_{50}$ with its standard error estimated after the probit method. The magnitude and significance of any differences between the control C3H series and the experimental hybrid groups could be estimated on the analogy of a six-point assay.

The second experiment utilised available "takes "arising from these attenuated inocula, 24 tumours in FHC and 32 in FFC mice, which were then treated *in situ* at three dose-levels in each genetic subgroup. Since it had been previously shown that the magnitude of the attenuation dose, within the range used in this investigation, had little, if any effect on the subsequent response to treatment, all "takes" were pooled, irrespective of the attenuation dose given, and redistributed for the purpose of the second experiment among the three dose-levels to be tested

TABLE I.—*Attenuation of C3H Tumour Prior to Implantation in Hybrid Hosts.*

| Dose (r). | FFC. Number of mice. | FFC. Number of takes. | FFC. Non-takes (%). | FHC. Number of mice. | FHC. Number of takes. | FHC. Non-takes (%). | Total Hybrids Takes. | Total Hybrids Non-takes (%). | Total C3H controls Takes. | Total C3H controls Non-takes (%). |
|---|---|---|---|---|---|---|---|---|---|---|
| 2000 | 9 | 9 | 0 | 10 | 10 | 0 | 19/19 | 0 | 44/44 | 0 |
| 2500 | 28 | 21 | 25 | 16 | 16 | 0 | 37/44 | 16 | 32/33 | 4 |
| 3000 | 20 | 3 | 85 | 20 | 10 | 50 | 13/40 | 68 | 7/25 | 72 |
| $LD_{50}$ | | 2700 (±70) r | | | 3000 (±100) r | | 2800 (±50) r | | 2800 (±60) r | |

*in situ*. The proportion of cures in each group was tabulated (Table II), and the $LD_{50}$ with its standard error estimated by the probit method for each genetic category. Using the previously reported series, in which unattenuated tumour was irradiated in both groups of hybrid hosts, as controls, the magnitude and significance of the attenuation effect in hybrids, both with and without the milk factor, could be estimated using standard methods of probit aanlysis.

TABLE II.—*Results of Treatment of Attenuated C3H Tumour in Hybrid Hosts.*

| Dose *in situ* | FFC. Number of mice. | FFC. Number cured. | FFC. Cures (%). | FFC. Controls. Cures. | FFC. Controls. (%). | FHC. Number of mice. | FHC. Number cured. | FHC. Cures (%). | FHC. Controls. Cures. | FHC. Controls. (%). |
|---|---|---|---|---|---|---|---|---|---|---|
| 3000 | 8 | 0 | 0 | — | 0 | 8 | 0 | 0 | — | 0 |
| 3500 | 12 | 5 | 42 | 2/11 | 18 | 12 | 1 | 8 | 0/11 | 0 |
| 4200 | 12 | 9 | 75 | 13/17 | 76 | 14 | 6 | 43 | 5/17 | 29 |

While a significant result in this comparison would prove the existence of the attenuation effect sought, an insignificant result would not, of course, prove that such an effect was absent. However, an estimate of the relative radiosensitivity of attenuated homoplasts in C3H hosts compared to controls, a ratio measuring the attenuation effect in the parent strain, is available and its standard error is known (Cohen and Cohen, 1954*b*), so that it also becomes possible to compare the magnitude of the attenuation effect, if any, in the hybrids with that in the parent strain (Table III).

TABLE III.—*Induced Radiosensitivity with Attenuated Homoplasts in Various Recipient Hosts.*

| Recipient Host. | Pre-treatment of tumour. | $LD_{50}$ (r). | Standard error of $LD_{50}$ | Relative acquired Radiosensitivity. |
|---|---|---|---|---|
| C3H | Controls | 5700 | ± 140 | 1·27 (±·07) |
| | Attenuated | 4500 | ± 200 | |
| FHC | Controls | 5100 | ± 400 | 1·17 (±·12) |
| | Attenuated | 4350 | ± 220 | |
| FFC | Controls | 3950 | ± 110 | 1·08 (±·06) |
| | Attenuated | 3600 | ± 200 | |

## RESULTS.

The results of irradiation of tumour fragments *in vitro* prior to implantation in hybrid recipient hosts is shown in Table I together with the C3H control series. From these findings the $LD_{50}$ appears to be 2700 ($\pm$70) r in the FFC and 3000 ($\pm$100) r in the FHC group. These two values do not differ significantly from each other or from the C3H controls; the $LD_{50}$ for the pooled hybrids was 2800 ($\pm$50) r which is virtually identical with that for the C3H control series 2800 ($\pm$69) r.

Contrary to the situation existing in the case of irradiation of the established tumour *in situ* therefore the response of the C3H tumour to an "attenuation dose" delivered before implantation is not obviously affected by genetic or extra-chromosomal factors in the susceptible hosts inoculated. Mice in both groups in whom attenuated inocula had not taken, exhibited no overt immunity when subsequently challenged with unattenuated tumour.

Cure rates obtained when "attenuated takes" resultant from the preceding experiment were subsequently irradiated *in situ* are shown in Table II together with previously reported data on the response of non-attenuated tumours in the corresponding hosts. On the whole, the percentage of cures in the attenuated series seems somewhat greater than that of the controls, the difference being more marked in the case of the FHC group. For the purpose of statistical analysis the $LD_{50}$'s and their standard errors for both attenuated and control tumours in both hybrid groups and in the C3H parent strain are shown in Table III.

It will be noted that the relative increase in radiosensitivity induced by the attenuation procedure is maximal in the C3H host (by a factor of 27 per cent which is significant at $p = \cdot0001$) is smaller in the FHC host (17 per cent which is not statistically significant) and is least in the FFC host (where the increase is only 8 per cent and statistically insignificant). While the existence of induced radiosensitization in the C3H mouse therefore is unequivocal and a similar but smaller effect may possibly exist in the factor-harbouring hybrid (in which the relatively large variation precludes conclusive analysis) the attenuation effect is significantly smaller, possibly entirely absent, in the factor-free hybrid host.

There appears, therefore, an interesting inverse relationship in that whenever an efficient genetically-conditioned resistance operates so as to increase the radiosensitivity of the homoplast, little or no additional radiosensitization can be induced by the attenuation procedure, while conversely, where genetic resistance is less efficient, induced radiosensitization becomes more effective.

## DISCUSSION.

In previous reports it had been shown that the radiosensitivity of a tumour treated *in situ* varies according to the degree of resistance of the host; the $LD_{50}$ being significantly lower in hybrid mice bearing the parent strain tumour than in the C3H parent host, and lower, too, in the absence of the milk-factor than in factor-harbouring hosts. The $LD_{50}$ in the C3H mice was also significantly lowered if the homoplast was " attenuated " by treatment with a sublethal but sufficient dose of radiation immediately prior to implantation. By contrast, the present series of experiments gave essentially negative results. When tumour fragments were irradiated before implantation, the $LD_{50}$ was apparently identical in the

parent strain host and in both hybrid subgroups, though still much smaller than the curative dose *in situ*, whether in C3H or hybrid hosts. These facts suggest that an irradiated tumour fragment is subjected at the time of implantation to a reaction on the part of the host of a certain maximal intensity which cannot be further enhanced by immunogenetic processes in the susceptible hybrid host.

When the attenuated "takes" in the two hybrid groups were subsequently treated *in situ*, the degree of induced radiosensitization encountered, instead of being large and highly significant as previously observed in the C3H host, was smaller and statistically insignificant. This effect was also apparently less marked in factor-free hybrids than in factor-harbouring mice. It thus appears that the greater the immunogenetically-conditioned radiosensitivity, the less the possibility of further attenuation-induced radiosensitization. It seems probable that both the genetic resistance and the reaction to attenuated implants affect the radiosensitivity of the homoplast through a common underlying mechanism, probably in the nature of the circulating antibodies demonstrated by Gorer (1947) and later by Fink, Snell and Kelton (1953). The combination of the two factors, therefore, would have limited, if any, additive effects. The convergent trend resulting from combinations of factors increasing host-resistance is shown in Fig. 1.

The differences between the reciprocal hybrid groups reflect the action of a maternal influence, here presumed to be identical with the milk-factor. In all

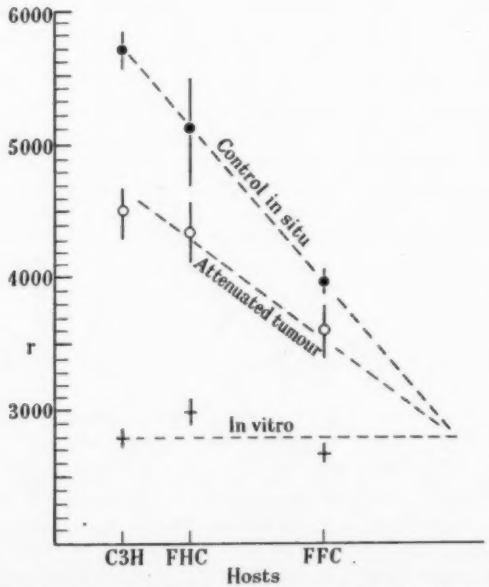


FIG. 1.—Diagram showing the median lethal doses (scale on left) and their standard errors (vertical lines) for the C3H carcinoma irradiated in various situations. Abscissae: Degrees of genetic difference between host and tumour arbitrarily spaced so as to emphasise the convergent trend towards lower effective dosage as increased host-resistance is evoked.

experiments testing immunogenetic differences, the factor-free hybrid diverges most from the C3H parent, while its factor-harbouring counterpart consistently shows an intermediate and somewhat more variable effect. The milk-factor, acting on the infant hybrid mouse, thus seems partially and inconstantly to obliterate immunogenetic differences, virtually inducing an acquired tolerance to the tumour, analogous to the "actively acquired tolerance to foreign cells" described by Billingham, Brent and Medawar (1953) in CBA mice.

The results of the foregoing series of experiments lend strong and consistent support to the hypothesis that the radiosensitivity of a tumour is a function of a specific systemic host resistance against that tumour. This single assumption suffices to explain four observations bearing upon the problem:

(1) That the tumour growing in hybrids is more radiosensitive than in parent-strain hosts;
(2) the milk-factor, tending to obliterate immunogenetic reactions to the tumour, reduces its radiosensitivity;
(3) tumours arising from radiation-attenuated implants in the C3H host are more radiosensitive (" induced radiosensitization");
(4) whole body irradiation, by deranging the resistance mechanism of the host, reduces the radiosensitivity of the tumour (Cohen and Cohen, 1953*b*).

If, as seems indicated by the present investigation, an irradiated tumour fragment is subjected at the time of implantation to a reaction on the part of the host approaching a certain well-defined maximum limit of intensity, and mediated through the same underlying mechanism as immunogenetic resistance, then the following three additional observations are also seen to fit the original hypothesis:

(5) The lethal dose for irradiation prior to implantation is smaller than that required by the established tumour *in situ*;
(6) the lethal dose for irradiation prior to implantation is not affected by genetic or maternal factors;
(7) radiosensitization induced by prior attenuation is less marked in hybrid hosts.

## SUMMARY.

When the C3H mammary carcinoma was irradiated before implantation into reciprocal $F_1$ hybrid hosts, the $LD_{50}$ was found to be 2700 r in (CBA × C3H)$F_1$ mice, presumably factor-free, and 3000 r in the factor-harbouring (C3H × CBA)$F_1$ reciprocal. These values do not differ significantly from each other or from that of the same tumour in the C3H parent-strain host (2800 r).

Whereas tumours arising from radiation-attenuated, viable implants in the parent strain had shown an increased radiosensitivity when subsequently treated *in situ*, the median lethal dose for similarly attenuated tumours in $F_1$ hybrid hosts did not differ significantly from non-attenuated homoplasts. The factor-harbouring hybrid, however, bore a closer resemblance to the C3H parent, showing a somewhat greater tendency to induced radiosensitization than the factor-free reciprocal.

It is concluded that radiosensitivity is a function of host resistance which may be enhanced both by inoculation of radiation-attenuated implants, and by immu-

nogenetic differences between the heterozygous hybrid host and the homozygous tumour, but a combination of both procedures, which presumably act through the same underlying mechanism, has no obvious additive effect.

We are indebted to Dr. J. F. Murray for providing facilities at the South African Institute for Medical Research where the C3H/Cg mouse colony is maintained. We are also grateful to Mrs. Shelley Jacobson for invaluable skilled assistance in the upkeep and breeding of this colony.

## REFERENCES.

Billingham, R. E., Brent, L., and Medawar, P. B.—(1953) *Nature*, **172**, 603.
Cohen, A., and Cohen, L.—(1953*a*) *Brit. J. Cancer*, **7**, 231—(1953*b*) *Ibid.*, **7**, 452.—(1954*a*) *Ibid.*, **8**, 303.—(1954*b*) *Ibid.*, **8**, 313.
Fink, M. A., Snell, G. D., and Kelton, D.—(1953) *Cancer Res.*, **13**, 666.
Gorer, G.—(1947) *Ibid.*, **7**, 634.

# EFFECT OF DITHIOCARBAMATES ON SARCOMA CELLS AND FIBROCYTES CULTURED *IN VITRO*.

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COMPOUNDS belonging to the carbamate series, notably ethylcarbamate (urethane), show pronounced biological activity. In addition to being narcotics, carbamates induce pulmonary tumours in mice (Nettleship and Henshaw, 1943; Henshaw and Meyer, 1945; Larsen 1946, 1947). Urethane inhibits tumour growth in experimental animals (Haddow and Sexton, 1946) causes a decrease of normal and malignant leucocytes (Paterson, Haddow, Ap Thomas and Watkinson, 1946), and inhibits mitosis (Lasnitzski, 1949; Hughes, 1950). In the dithiocarbamate series sulphur replaces the oxygen of the carbamic acid radicle. In view of this relationship the effects of piperidinium pentamethylene dithiocarbamate ($C_5H_{10}NCSSNH_2C_5H_{10}$), sodium pentamethylene dithiocarbamate ($C_5H_{10}NCSSNa$), and sodium diethyldithiocarbamate ($(C_2H_5)_2NCSSNa$), on the growth of sarcoma cells and fibrocytes cultured *in vitro* have been studied. The main purpose of this work was to find out whether these reagents had any selective action on the growth and viability of malignant cells.

Each method used in screening compounds for possible chemotherapeutic activity against malignant tumours has certain advantages and limitations, but cultured cells provide suitable test material for making preliminary comparative studies upon malignant and normal cells. *In vitro* trials have the particular advantage that a reagent can be kept in prolonged contact with cells at a relatively constant concentration and is not metabolized by tissues other than those under test. On the other hand, normal cells undergo pronounced morphological and physiological changes when actively growing *in vitro* and are not strictly comparable to the differentiated cells of an intact animal. Moreover distinctions between malignant and normal cells in growing cultures are less evident *in vitro* than *in vivo*.

For this reason investigations of the cytotoxic effects of the dithiocarbamates have been made both on cells cultured in a state of functional survival with minimal proliferation and on actively growing and dividing cells. The methods adopted were the cultivation of tissue fragments suspended in fluid media and by the double-coverslip method of Maximow, respectively.

## MATERIALS AND METHODS.

The cultivated tissues were taken from rapidly growing transplanted homologous spindle-celled sarcomas maintained in CBA strain mice and, as control material, thigh muscle from 14–17 day CBA embryos which gives luxuriant outgrowths of fibrocytes.

Double-coverslip cultures were prepared as follows : suitable pieces of tissue were prepared in a mixture of 60 per cent balanced saline solution (Earle, 1943) and 40 per cent horse serum, mounted in a coagulum consisting of one drop of mouse embryo extract added to one drop of a mixture of equal parts of fowl and rat plasma. After coagulation of the plasma clot one drop of nutrient medium was placed over the explant. This medium consisted of 2 parts horse serum, 2 parts Earle's solution containing phenol red and adjusted to pH 7·4–7·6, and one part embryo extract.

At least two preliminary subcultures were made before adding the drug solutions. These were prepared in Earle's solution, sterilized by passage through Seitz filters and incorporated in the nutritive medium. Media for control solutions received Earle's solution alone in equal amounts. Test solutions were added 24 hours after previously washing and re-feeding the cultures in order to ensure active growth and division of the cells at the time of application.

In each experiment, 6 cultures were exposed to each of the range of drug concentrations. Cultures were inspected and fixed in Susa solution for staining with Harris's haematoxylin 24 hours after applying a drug when mitoses are abundant in control cultures. Cultures were fixed also after 48 and 72 hours, treatment.

In the fluid cultures, 6 fragments of tissue 1 mm.$^3$ in size were cultivated together in 12 drops of nutritive medium, as used in the double-coverslip cultures, in large cavity slides covered with mica slips sealed with paraffin wax.

After 48 hours' exposure to the drug solutions pieces of tissue were removed from the culture medium, washed thoroughly in Earle's solution and explants cultured singly, by the double-coverslip method, in normal media. In each experiment, 6 cultures were prepared from tissue fragments exposed to each drug concentration. Similar material from each series was mounted in plasma clots and fixed in Susa for the preparation of sections stained with Ehrlich's haematoxylin and eosin. Examination of the double-coverslip cultures permitted the estimation of the maximum concentration of a reagent which the cells could tolerate for 48 hours and the minimum lethal dose.

Preliminary assays of the dithiocarbamates were made to determine their working ranges.

## EXPERIMENTAL RESULTS.

All experimental procedures were standardized but there were inherent variations in, for example, the nutritive qualities of the plasma and embryo extract used in different experiments. In each experimental series one drop of drug-containing medium was added from the same pipette to all cultures to decrease differences in the volume of drug solution. The relative concentration of the reagents were maintained as between different series of tests.

The morphological effects of the three drugs at toxic concentrations were similar in both normal and tumour cells. Healthy sarcoma cells were considerably larger than the more slender fibrocytes and had larger, more deeply stained nuclei. (Fig. 1 and 5). Treated cells of both types showed non-specific effects. Vacuolation of nuclei and cytoplasm, deformed and pyknotic nuclei and basophilic debris were commonly observed. The cells quickly rounded up when exposed to weak toxic dosages. Viability was assessed by standard morphological criteria and the development or absence of new outgrowth. The results are given in Tables I and II.

TABLE I.—*Effects of Dithiocarbamates on Viability of Cells of Culture Outgrowths.*

| Compound tested. | Cultured cells. | Controls. | Drug concentration. M/125,000. | M/100,000. | M/50,000. | M/25,000. | M/5,000. |
|---|---|---|---|---|---|---|---|
| Piperidinium pentamethylenedithiocarbamate | Sarcoma cells | ++++++ | ++++++ | ++±±±± | ±±±−−− | −−−−−− | −−−−−− |
| | Fibrocytes | ++++++ | ++++++ | ++++±± | ±±±±−− | −−−−−− | −−−−−− |
| Sodium pentamethylenedithiocarbamate | Sarcoma cells | ++++++ | ++++++ | ++++±± | ±±±−−− | −−−−−− | −−−−−− |
| | Fibrocytes | ++++++ | ++++++. | +++±±± | ++±−−− | −−−−−− | −−−−−− |
| Sodium diethyldithiocarbamate | Sarcoma cells | ++++++ | .. | ++++++ | +++±−− | −−−−−− | −−−−−− |
| | Fibrocytes | ++++++ | .. | ++++++ | +++++ | ++++±± | −−−−−− |

\+ = No effect. ± = Marked effect. − = All cells killed.

TABLE II.—*Effect of Dithiocarbamates on Development of Outgrowths from Fragments of Tissue Incubated in Fluid Media.*

| Compound tested. | Cultured tissue. | Controls. | Drug concentration M/125,000. | M/100,000. | M/50,000. | M/25,000. | M/5,000. |
|---|---|---|---|---|---|---|---|
| Piperidinium pentamethylenedithiocarbamate | Sarcoma cells | ++++++ | ++++++ | ++++++ | +++±±± | +++±±± | −−−−−− |
| | Fibrocytes | ++++++ | ++++++ | ++++++ | +++++ | ++++±− | −−−−−− |
| Sodium pentamethylenedithiocarbamate | Sarcoma cells | ++++++ | .. | ++++++ | −−−−−− | −−−−−− | −−−−−− |
| | Fibrocytes | ++++++ | .. | ++++++ | +−−−−− | −−−−−− | −−−−−− |
| Sodium diethyldithiocarbamate | Sarcoma cells | ++++++ | .. | ++++++ | ++++++ | −−−−−− | −−−−−− |
| | Fibrocytes | ++++++ | .. | ++++++ | ++++++ | −−−−−− | −−−−−− |

\+ = Outgrowth normal. ± = Outgrowth reduced in size. − = Outgrowth absent.

In chemotherapeutic trials *in vivo* positive selective action upon malignant cells depends on their being affected at drug concentrations which do not appreciably affect normal cells. The criteria of (*a*) the lowest concentration of a drug which (i) completely suppressed the development of outgrowth or (ii) killed all the cells of established outgrowths, in all 6 cultures of a set and (*b*) the highest concentration which (i) allowed the formation of a normal outgrowth or (ii) had no evident toxic effect on an established outgrowth, in each of the 6 cultures of a set, are probably more reliable standards for comparing the effects of the dithiocarbamates upon the test material than the variable results got at intermediate concentrations.

*Established outgrowths.*

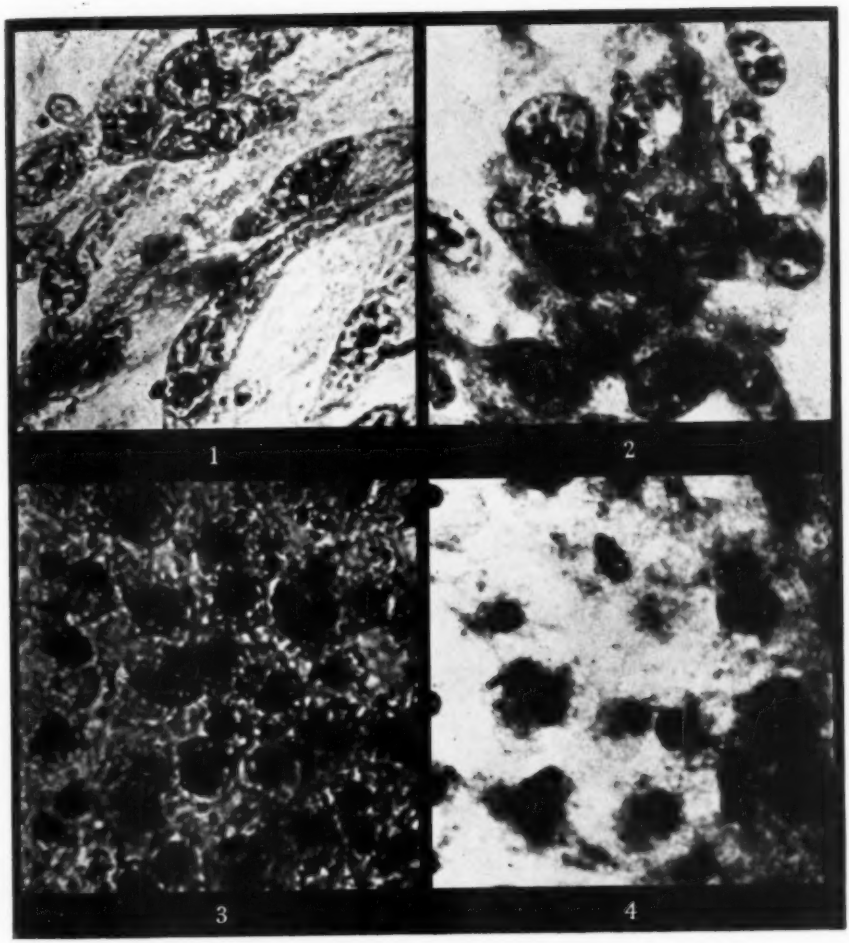
The toxicities of piperidinium- and sodium-pentamethylene dithiocarbamates were closely comparable. Both showed no evidence of toxicity at concentrations of M/125,00 and were lethal at M/25,000. There was no significant difference in the response of sarcoma cells and fibrocytes to either drug, although at intermediate concentrations sarcoma cells appeared slightly more sensitive than fibrocytes. Sodium diethyldithiocarbamate (Fig. 1–8) appeared to be less toxic than either of the preceding compounds possibly because of its simpler molecular structure. It was more toxic to sarcoma cells, being lethal at M/25,000, than fibrocytes, which were only slightly affected at this dosage, but killed at dosages of M/5,000.

*Fluid cultures.*

Drug toxicities were assayed by the presence or absence of outgrowth from fragments of tissue first incubated for 48 hours in the presence of the drug and by examination of stained sections of tissue fixed after the 48 hours' treatment. Outgrowths developed from all explanted fragments of both tumour and muscle previously exposed to M/100,000 dosages of all three compounds. No outgrowths developed from tissue fragments treated with M/25,000 sodium pentamethylene- and sodium diethyldithiocarbamates. Piperidinium pentamethylene dithiocarbamate appeared less toxic by this criterion. The results obtained by the two methods of assay agreed at drug concentrations of M/100,000 and M/5,000, that is, the invariably non-toxic and lethal dosages respectively. At intermediate dosages the presence in sections of cells seemingly normal by morphological

EXPLANATION OF PLATES

FIG. 1.—Sarcoma cells of outgrowth. Culture untreated.
FIG. 2.—Sarcoma cells of outgrowth. Culture treated with M/100,000 sodium diethyldithiocarbamate.
FIG. 3.—Sarcoma cells of outgrowth. Culture treated with M/25,000 sodium diethyldithiocarbamate.
FIG. 4.—Sarcoma cells of outgrowth. Culture treated with M/5,000 sodium diethyldithiocarbamate.
FIG. 5.—Fibrocytes of outgrowth. Culture untreated.
FIG. 6.—Fibrocytes of outgrowth. Culture treated with M/100,000 sodium diethyldithiocarbamate.
FIG. 7.—Fibrocytes of outgrowth. Culture treated with M/25,000 sodium diethyldithiocarbamate.
FIG. 8.—Fibrocytes of outgrowth. Culture treated with M/5,000 sodium diethyldithiocarbamate.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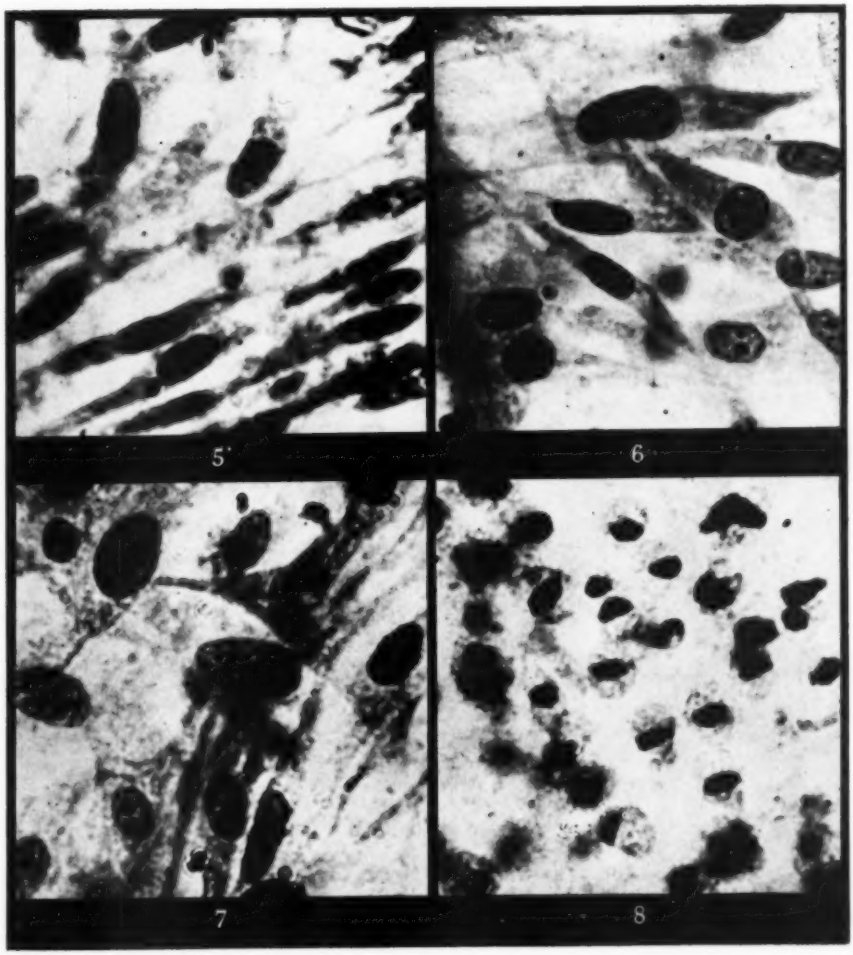

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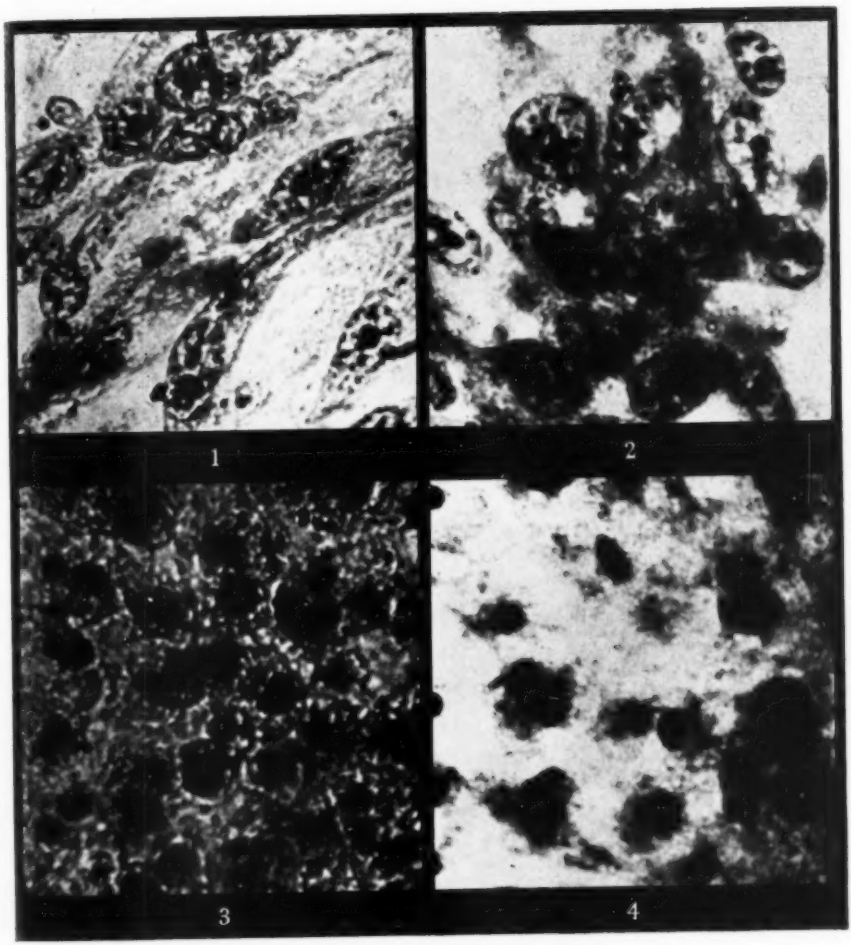


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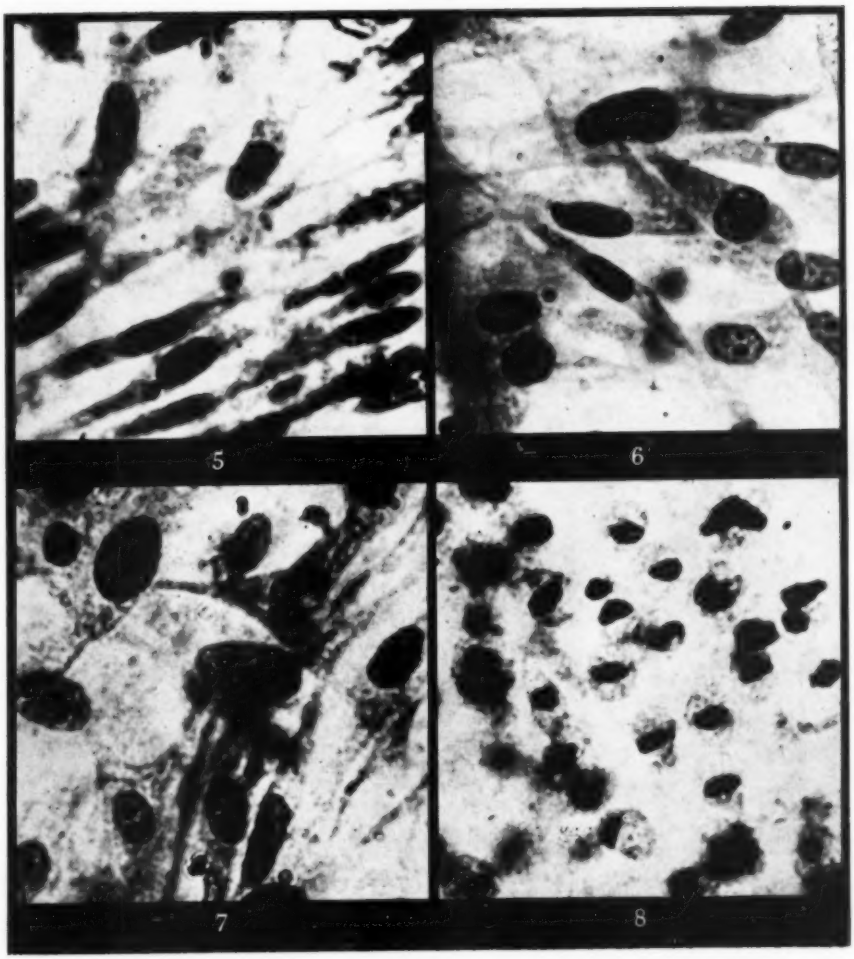

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criteria was not invariably paralleled by the development of outgrowth from equivalent tissue fragments similarly treated.

The pieces of cultured tissue varied somewhat in size. In control material normal cells were usually restricted to a peripheral zone some 12–15 cells thick. Unaffected cells internal to dead ones were often apparently unable to migrate and divide in subcultured explants. The fragments of tissue were not small enough to permit the survival of all the cells in control material and possibly the complete penetration by a drug in treated material. The tests on established outgrowths in which all the cells were exposed to the drugs were probably more reliable for assaying relative toxicities.

## DISCUSSION.

The greater toxicity of sodium diethyldithiocarbamate to sarcoma cells than fibrocytes in actively growing cultures may be related to its known properties. It serves as an ultra-accelerator in the vulcanization of rubber. Schraufstätter (1950) has reported it to be bactericidal to *M. tuberculosis*, *S. aureus* and *Salm. paratyphi* at concentrations of 1/32,000–1/8,000. It is unstable in aqueous media, liberating carbon disulphide. The free acid hydrolyzes to thiocyanic acid and hydrogen sulphide.

The diethyldithiocarbamate ion readily forms co-ordination compounds with many cations especially copper and is used for quantitative estimations of the latter. The sodium salt increases the stability of ascorbic acid in solution (Campbell and Tubb, 1950) and neutralizes the inhibitory effects of ascorbic acid-copper complex on phosphorylase (Sri Ram and Giri, 1949). Bodine and Fitzgerald (1948) reported that sodium diethyldithiocarbamate in low concentrations stimulated and at higher concentration first depressed and later stimulated oxygen uptake by both dividing and " blocked " cells of embryos of *Melanoplus differentialis*. They also found that it decreased the toxicity of copper acetate to the embryos and suggested that its effect on respiration might be linked with its ability to react with copper ions. Sulphydryl-containing compounds have an affinity for copper-containing enzymes (Barron and Singer, 1945).

Thus it appears that sodium diethyldithiocarbamate can affect oxidation-reduction systems and cellular respiration. These properties may be responsible in part for the reported greater sensitivity of sarcoma cells than fibrocytes in rapidly growing cultures.

## SUMMARY.

1. The toxicities of piperidinium pentamethylene-, sodium pentamethylene-, and sodium diethyldithiocarbamates to sarcoma cells and embryonic mouse fibrocytes cultured *in vitro* have been studied.

2. Sodium diethyldithiocarbamate is the least toxic of these compounds and is more toxic to sarcoma cells than fibrocytes in rapidly growing cultures.

3. It is suggested that this property may be related to the reported effects of this compound on oxidation-reduction systems and cellular respiration.

I am indebted to Monsanto Chemicals, Ltd., and Robson Bros. Ltd., West Bromwich for the gift of sample of piperidinium- and sodium-pentamethylene dithiocarbamate. My thanks are due to Mr. G. A. Butcher for his technical assistance.

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## REFERENCES.

BARRON, E. S. G., AND SINGER, T. P.—(1945) *J. biol. Chem.*, **157**, 221.
BODINE, J. H., AND FITZGERALD, L. R.—(1948) *Proc. Soc. exp. Biol., N.Y.*, **69**, 442.
CAMPBELL, J., AND TUBB, W. G.—(1950) *Canad. J. Res.*, **28E**, 19.
EARLE, W. R.—(1943) *J. nat. Cancer Inst.*, **4**, 165.
HADDOW, A., AND SEXTON, W. A.—(1946) *Nature*, **157**, 500.
HENSHAW, P. S., AND MEYER, H. L.—(1945) *J. nat. Cancer Inst.*, **5**, 415.
HUGHES, A. F. W.—(1950) *Quart. J. micr. Sci.*, **91**, 251.
LARSEN, C. D.—(1946) *J. nat. Cancer Inst.*, **7**, 5.—(1947) *Ibid.*, **8**, 99.
LASNITZSKI, I.—(1949) *Brit. J. Cancer*, **3**, 501.
NETTLESHIP, A., AND HENSHAW, P. S.—(1943) *J. nat. Cancer Inst.*, **4**, 309.
PATERSON, E., HADDOW, A., AP THOMAS, I., AND WATKINSON, J. M.—(1946) *Lancet*, i, 677.
SCHRAUFSTÄTTER, E.—(1950) *Z. Naturf.*, **56**, 190.
SRI RAM, J., AND GIRI, K. Y.—(1949) *Curr. Sci.*, **18**, 440.

# GROWTH CHARACTERISTICS OF THE ROUS NO. 1 SARCOMA GROWN IN THE PERITONEAL CAVITY OF CHICKENS.

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THE establishment and a description of the general properties of an ascites variant of the Rous No. 1 sarcoma have been discussed in a previous publication (Bather, 1954).

Growth of a tumour in the form of a cell suspension in the peritoneal cavity enables a direct study to be made of the increases in total tumour cells with time. This has been done for the Ehrlich and MCIM mouse ascites tumours by Klein and Révész (1953). Lasnitzki (1952) used ascites forms of S37 and T2146 mouse tumours to study the morphology and mitosis of the tumour cells throughout their growth period. The Rous ascites tumour provides the opportunity of comparing a virus induced tumour with the non-filterable mammalian ones so far studied in this respect.

## MATERIALS AND METHODS.

### *Preparation of standard inoculum.*

The animals used were from the flock of inbred brown leghorns maintained at this centre. Five-week old chickens of the Intensity × Breeding line were used; these birds are highly susceptible to the Rous sarcoma virus. Care was taken to choose birds of approximately equal size. A standard inoculum of 5 million tumour cells was prepared as follows. A bird bearing a well developed 14-day ascites tumour from the 27th passage was killed and as much ascites fluid as possible was removed aseptically with a glass micro-pipette fitted with a rubber bulb. A sample was immediately smeared, fixed in Susa and stained with Leishman stain. At the same time, a cell count was made of another sample of fluid in a haemacytometer. The remainder of the fluid was stored in the refrigerator until needed.

The cell count gave $25{\cdot}6 \times 10^6$ total cells/ml. From the stained slide, a differential count showed that 244/513 (48 per cent) of the cells were malignant Rous cells. Thus the fluid was now known to contain $12{\cdot}3 \times 10^6$ Rous cells/ml. and was diluted with saline to give a suspension containing $5 \times 10^6$ Rous cells/ml. This preparation was used to inoculate the chickens for a determination of the growth curve. A similar preparation containing 10 million cells was made and diluted serially to determine the minimum dose of tumour cells required to start an ascites tumour (Experiment 1).

### *Method of harvesting cells.*

At intervals after inoculation birds were killed and the total intraperitoneal cell content determined by a similar method to that suggested by Klein and

Révész (1953). To do this, the skin was removed from the abdomen and a small hole made in the body wall immediately adjacent to the right pubis. As much fluid as possible was removed with a glass pipette and bulb. Ringer's solution was then introduced into the body cavity in approximately 5 ml. amounts and circulated about by tilting the carcass. The Ringer washing was then removed by means of the glass pipette and bulb. This operation was continued until the washings became quite clear and no further cells could be removed. The volume of the total fluid and washings was then measured and a cell count done in a haemacytometer. A sample of the harvest was centrifuged to deposit the cells which were then taken up in a small quantity of 5 per cent gelatin in physiological saline for smearing and staining. A differential count was done which, in conjunction with the total cell count, gave an estimate of the total tumour cell content of the bird.

The mitotic index for each tumour was estimated for a 500-cell sample from the stained smears. Invasion of internal organs was followed by taking sections of heart, lungs, liver, spleen, pancreas, duodenum, kidney and ovary or testis from each bird.

## EXPERIMENTAL.

(1) *Determination of the number of tumour cells required to establish the ascites tumour.*

The tumour cell content of a sample of ascites fluid taken on the 10th day of growth was determined as outlined. Serial tenfold dilutions were made in saline so that a series of suspensions containing from $10^2$–$10^7$ Rous cells were obtained. Five birds were used for each dilution and each bird received 1·0 ml. of the suspension intraperitoneally. The results are summarized in Table I. Included in the table are 5 birds which received 1·0 ml. of a cell-free preparation of ascites fluid containing $10^4$ M.I.D.'s per ml. of Rous sarcoma virus (determined by titration in day old chicks— Carr and Harris, 1951). The final column gives the range of survival times for the birds of each group.

TABLE I.—*Incidence of Ascites Growth and Chicken Survival Time after the Intraperitoneal Inoculation of Rous Ascites Cells or Virus.*

| Tumour cells inoculated. | Ascites growth | Survival time (days) |
|---|---|---|
| $10^7$ | 5/5 | 9–11 |
| $10^6$ | 5/5 | 10–11 |
| $10^5$ | 5/5 | 11–14 |
| $10^4$ | 5/5 | 15–21 |
| $10^3$ | 4/5 | 15–17 |
| $10^2$ | 2/5 | 18–19 |
| Cell-free virus | 0/5 | — |

From Table I it is apparent that as few as 100 tumour cells are sufficient to establish the Rous ascites tumour. Cell-free virus, on the other hand, does not give rise to ascites formation, although 2 birds of this group grew subcutaneous tumours at the site of inoculation. These results serve to emphasize the highly malignant properties of the Rous sarcoma cells. The final column of the table

shows that the larger the initial inoculation the shorter is the survival time of the host.

(2) *Growth curve of the Rous ascites tumour.*

When estimating the intraperitoneal cell content by the flushing technique, the measurements decrease markedly in accuracy if the numbers of cells to be counted fall below about $10^6$ in an animal as large as a 5-week old chicken. It was decided, therefore, to use an initial inoculum of $5 \times 10^6$ Rous cells in the growth study presented in the next experiment. A group of 40 5-week-old chickens were each inoculated with 1·0 ml. of the suspension prepared as described and containing $5 \times 10^6$ Rous cells/ml. Total tumour cell contents estimated as outlined previously were determined at intervals after inoculation and are plotted as log tumour cell numbers in the graph in Fig. 1. Each point on the graph represents the mean of 3 determinations with the exception of the 3rd and 13th days. At the 3rd day only 2 birds were used and at the 13th day only 1 remained alive. The first death occurred at 11 days and by the 13th day, all the remaining animals but one had died.

When calculating the best curve to fit the observed points it was decided to include all observations made after 0 time. As shown in Fig. 1 there was a fall in tumour cell numbers during the first 24 hours and the stained smears made during the first 3 days showed numerous giant macrophages containing phagocytosed cells and cell fragments. This reaction of the bird to the tumour inoculation and the tendency for the inoculated cells to aggregate at certain sites in the peritoneal cavity (see Experiment 5) probably account for the greater part of the initial fall in tumour cell numbers. Blood smears made during the first 3 days showed no evidence of a migration of malignant cells to the circulating blood.

A best straight line fitted to all the points from the 1st to the 13th days was found to conform to the regression equation $y = 0{\cdot}233 + 6{\cdot}37$ (Fig. 1—solid line). Analysis of variance revealed that the variation due to regression was very highly significant. Table II shows that the mean square of the deviations from regression is small but not significantly so when compared to the error mean square.

TABLE II.—*Analysis of Variance of the Deviation from the Regression Equation* $y = 0{\cdot}233 + 6{\cdot}37$.

| Source of Variation | Degree of freedom. | Mean squares. |
|---|---|---|
| Regression | 1 | 19·5410 |
| Deviation from Regression | 9 | 0·0571 |
| Error | 19 | 0·0949 |

The numbers of tumour cells increase logarithmically with time and reach a maximum of approximately 2500 million in 13 days in a bird inoculated at 5 weeks of age. There was no justification for placing anything but a straight line relationship between time and the log of the tumour cell numbers on the present set of results and no dying away of the growth curve was apparent towards the end.

(3) *Variation in the percentages of tumour and non-tumour cells with time.*

The percentages of tumour cells each day were calculated by differential counts of stained smears and are plotted in Fig. 2. The percentage at time 0 did not cor-

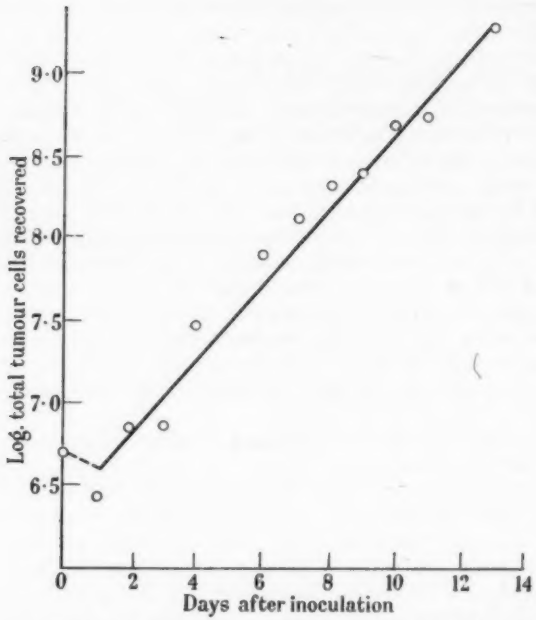


FIG. 1.—Total ascites tumour cells recovered at various intervals after the intraperitoneal inoculation $5 \times 10^6$ tumour cells.

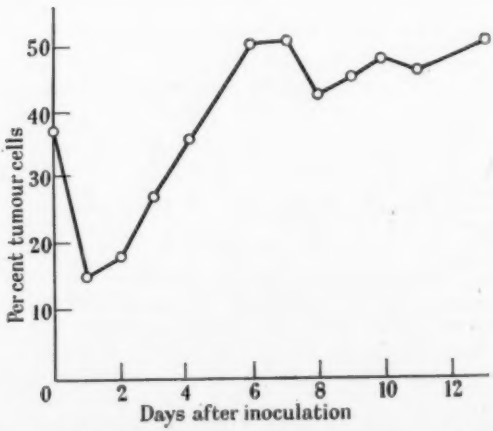


FIG. 2.—Percentage tumour cells in the total free ascites cells recovered at intervals after the intraperitoneal inoculation of $5 \times 10^6$ tumour cells.

respond to that of the inoculum because of the normal occurrence of intraperitoneal cell-containing fluid in small amounts. Five normal 5-week old chickens were used to determine the average number of free intraperitoneal cells by the flushing technique already described. It was found that an average of $3{\cdot}1 \times 10^6$ must be added to the inoculated number of cells in order to obtain a true estimate of the percentage of intraperitoneal tumour cells at 0 time (Fig. 2).

From Fig. 2 it can be seen that there was a sudden drop in the relative number of tumour cells owing to the response of the host to the inoculum by mobilizing its defences against the new invasion, and the aggregation of tumour cells into small attached foci of malignant cells in the vicinity of the pancreas and duodenum. (Experiment 5). Large numbers of lymphocytes, leukocytes and connective cells made their appearance and it was not until the 4th day that the percentage of tumour cells approached that at 0 time. After this there was a rapid increase to the maximum of about 50 per cent tumour cells which remained fairly constant to the end. This tumour never reaches the " nearly pure culture " stage of the mouse ascites tumours, which attain 70–90 per cent malignant cells (Klein and Révész 1953). The data show, however, that from the 6th day on the increase of tumour cells and non-tumour cells go hand in hand, roughly equal numbers of each being produced.

(4) *Mitosis and mitotic cycle during the growth of the Rous ascites tumour.*

Again using stained smear preparations it was possible to determine the per cent mitosis, in both the suspended cells and the solid invasive tissue throughout the growth of the ascites tumour. Total mitoses included recognizable prophase right through to separation of the daughter cells. In this regard it might be mentioned that cells were often observed in telophase after failure of cleavage of the cell. This seemed to be the main cause of abnormal mitosis apart from chromosome stickiness. The percentage mitosis for both cells and solid tissue are plotted in Fig. 3.

It is obvious that the mitotic index of the free cells remains fairly constant throughout the growth cycle except for a rise at the beginning and a fall at the end.

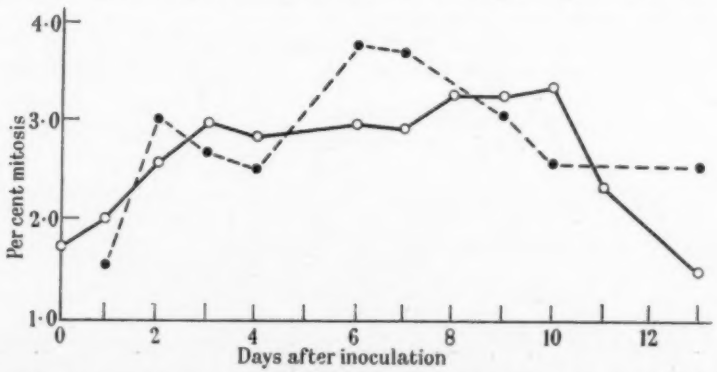


FIG. 3.—Mitotic index in the free ascites tumour cells and solid tumour tissue at intervals after the inoculation of $5 \times 10^6$ tumour cell.
○———○ Free tumour cells. ●– – – –● Solid tissue.

That of the solid Rous tissue appears more erratic but shows the same rise and fall. In describing the Rous ascites tumour in a previous paper (Bather, 1954) it was found that the average mitotic index was about 1·5 per cent. It is now seen that this is a low figure encountered only when the tumour growth is near or at its end. During the last day or two of tumour growth the bird becomes moribund and it is obvious that its whole metabolism is upset. A truer average figure for the mitotic index would be 2·7 per cent.

It is possible now to estimate the average length of the mitotic cycle of the freely suspended cells provided certain assumptions are made. First it is assumed that the logarithmic relationship between tumour cell numbers and time is true and that the straight line drawn represents the true increase of cell numbers with time. The mean generation time for the tumour cell population can then be calculated from the equation

$$g = \frac{T_2 - T_1}{\log N_2 - \log N_2} \times \log 2 \quad . \quad . \quad . \quad . \quad (1)$$

where $g$ = mean generation time, $N_2$ = number of viable cells at time $T_2$ and $N_1$ = number of viable cells at time $T_1$. Secondly, it is assumed that there is no increase in percentage cell death with time. No such effect has been found with either S37 or the Ehrlich ascites tumour when using special staining techniques (Klein and Révész, 1953). The nature of the ascites tumour growth is such that, in all probability, there are plenty of nutrients available at all times for all the cells.

Thirdly, the assumption is made that the majority of the tumour cells can undergo mitosis during the entire growth cycle and that " g " represents the average total length of one interphase + mitosis. The average mitotic time, or length of mitoses can be calculated from Crick's formula (quoted by Hughes, 1952). The formula relates the intermitotic period to the proportion of cells in mitosis as follows:

$$\frac{\text{Time of mitosis}}{\text{Total time of mitosis + interphase}} \times \log_e 2 = \log_e \frac{1 + 2R}{1 + R} \quad . \quad . \quad (2)$$

where $R$ is the fraction of cells in mitosis.

If the intermitotic period is relatively long, R is thus small and the right hand side of the equation approximates to R. The equation then becomes

$$\frac{\text{Time of mitosis}}{\text{Mean generation time}} = 1{\cdot}44R \quad . \quad . \quad . \quad . \quad (3)$$

From the Equation (1) the mean generation time for the logarithmic phase of the growth curve is 31 hours. Using this value and Equation (3) the mitotic time has been calculated for each day from the 1st–13th days of growth (Table III).

Provided the assumptions regarding the logarithmic increase in tumour cell numbers, the low incidence of cell death and the participation of the majority of cells in mitosis are correct, Table III gives a reasonable estimate of the mitotic time for Rous sarcoma cells grown in the form of an ascites tumour. The average time of mitosis is 73·6 minutes. No previous data for avian ascites tumours are available for comparison with this figure. Hughes (1952) quotes the mitotic time of normal proliferating chicken cells as 34–52 minutes.

TABLE III.—*Time of Mitosis each Day During the Growth of the Ascites Tumour Calculated for a Mean Generation Time of* 31 *Hours.*

| Time after inoculation (days). | Mitotic index. | Time of mitosis (minutes). |
|---|---|---|
| 1 | 2·00 | 53·6 |
| 2 | 2·60 | 69·7 |
| 3 | 3·00 | 80·5 |
| 4 | 2·84 | 76·1 |
| 6 | 3·02 | 81·0 |
| 7 | 2·90 | 77·7 |
| 8 | 3·31 | 88·7 |
| 9 | 3·27 | 87·7 |
| 10 | 3·39 | 91·0 |
| 11 | 2·35 | 63·0 |
| 13 | 1·50 | 40·2 |
| | Average = 2·74 | 73·6 |

(5) *Host tissue invasion during growth of the Rous ascites tumour.*

Sections of heart, lungs, liver, spleen, ovary or testis, adrenal, peritoneum, pancreas and duodenum were made from most birds throughout the growth experiment. The sections were fixed in Susa and stained with haematoxylin and eosin in order to determine the course of tumour invasion of the host tissues. Table IV shows the progressive invasive tendencies of the tumour. In this table, + indicates proliferating nodules of attached tumour tissue but no infiltration into the organ. ++indicates actual invasion and replacement of normal host tissue.

From the Table IV it is apparent that proliferation of tissue in the solid form starts very soon after the initial inoculation of Rous cells. After only 24 hours the peritoneum and the pancreas are providing the supporting surface for Rous growth. By 6 days the peritoneum, pancreas, duodenum spleen and ovary are all being invaded and the occurrence of solid tumour tissue is already becoming widespread. At 9 days, almost all the organs examined were involved to a greater or lesser degree. It seems that the establishment of the solid tumour takes place at an earlier stage in the growth of the ascites tumour than is the case with the mouse tumours studied by Klein and Révész (1953). It is highly probable that the growth of Rous sarcoma in the ascites form depends on the successful establishment of these foci of solid growth.

## DISCUSSION.

The experiments reported here enable the observer to follow the growth of the Rous No. 1 sarcoma more closely than has hitherto been possible, although admittedly under different conditions from those prevailing in the usual muscular inoculation. The cell counts during the first few days of growth show fairly conclusively that the final tumour cells are direct descendants of those inoculated.

The initial fall in the number of Rous cells recoverable by the flushing technique can be explained by host cell phagocytosis and tumour cell aggregation at suitable sites such as the peritoneum and the surface of the pancreas. This tendency of Rous ascites cells to aggregate rapidly has been noticed whenever ascites fluid has been removed and placed in a test tube or on a slide for microscopic examin-

TABLE IV.—*Tumour Tissue Attachment and Invasion of Host Organs following Intraperitoneal Inoculation of* $\times 10^6$ *Rous Cells.*

| Time after inoculation (days). | Bird No. | Peritoneum. | Pancreas. | Duodenum. | Spleen. | Sex Gland. | Liver. | Adrenal. | Heart. | Lungs. |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | — | — | — | — | — | — | — | — | — |
| | 2 | + | + | — | — | — | — | — | — | — |
| | 3 | + | + | — | — | — | — | — | — | — |
| 2 | 1 | + | + | — | — | — | — | — | — | — |
| | 2 | + | + | — | — | — | — | — | — | — |
| | 3 | + | + | — | — | — | — | — | — | — |
| 3 | 1 | + | + | — | — | — | — | — | — | — |
| | 2 | ++ | + | — | — | — | — | — | — | — |
| 4 | 1 | ++ | + | + | — | — | — | — | — | — |
| | 2 | ++ | + | ++ | — | — | — | — | — | — |
| | 3 | — | — | — | — | — | — | — | — | — |
| 6 | 1 | ++ | ++ | ++ | ++ | ++ | — | — | — | — |
| | 2 | ++ | — | ++ | — | — | — | — | — | — |
| | 3 | ++ | ++ | ++ | ++ | ++ | — | — | — | — |
| 7 | 1 | ++ | ++ | ++ | ++ | + | + | — | — | — |
| | 2 | ++ | ++ | ++ | ++ | + | — | ++ | — | — |
| | 3 | ++ | ++ | ++ | ++ | + | + | — | — | — |
| 9 | 1 | ++ | ++ | ++ | ++ | ++ | + | ++ | + | + |
| | 2 | ++ | ++ | ++ | ++ | ++ | + | + | — | + |
| | 3 | ++ | ++ | ++ | ++ | — | — | + | — | — |
| 10 | 1 | ++ | ++ | ++ | ++ | ++ | + | + | + | + |
| | 2 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | — | — |
| | 3 | ++ | ++ | ++ | ++ | + | — | + | — | — |
| 12 | 1 | ++ | ++ | ++ | ++ | ++ | — | + | + | + |

\+ indicates the presence of proliferating nodules of tumour cells attached to the organ or tissue involved but without infiltration.

++ indicates actual invasion and replacement of the host organ or tissue.

ation. It is likely that such aggregations become the first foci of tumour growth and are necessary for successful formation of the ascites tumour. A minimum tumour-inducing dose of 100 cells is in contrast with the findings of Fränkel (1927) who stated that 500,000 Rous cells were necessary to produce a tumour. The fact that the more cells injected, the quicker the growth of the tumours (Fränkel, 1927) finds confirmation in the ascites tumour (Table I). Klein and Révész (1953) found that approximately $0{\cdot}7 \times 10^6$ cells were necessary to produce 100 per cent takes of the Ehrlich ascites tumour in $C_3H$/St. mice. The high rate of survival and rapid growth of the Rous ascites tumour may be due to the infective virus which, by conferring highly malignant properties on the majority of tumour cells, may ensure the lack of any marked foreign body reaction.

The growth of the Rous tumour intraperitoneally recalls its behaviour in tissue culture. The original fragment cultured in a plasma-embryonic extract medium soon gives rise to migrating cells. Both spindle (fibroblast) and round (basophilic) cells together form a compact growing area which then begins to liquify the plasma clot. The cells split up and with in 24 hours the original fragment is surrounded

by a large liquified area, abounding in round cells (Tenenbaum, 1943). Tenenbaum (1943) and co-workers believe the round and spindle cells to be different forms of the same mesenchyme cell and have observed many intermediate steps in the reversible transformation. The cell itself, by its proteolytic powers, is able to create the conditions required for its diversity of form. It is very likely that the intensive invasion of the normal tissues is largely brought about by liquefaction of the normal cell cement substance by the sarcoma cells.

After the initial latent period of 24 hours the increase in tumour cell numbers with time follows the logarithmic law fairly closely with little or no "die-away" tendency towards the end. This is probably due to the early death of the bird as well as the lack of foreign body reaction. Rous tissue invasion of the internal organs is extremely rapid and extensive. In the terminal stages of tumour growth, the pancreas and ovary are almost completely replaced and duodenal invasion has led to the disintegration of the duodenal wall. The host, therefore, probably succumbs before the concentration of free tumour cells has become a limiting factor to their growth. The progressively increasing generation times observed in the Ehrlich and MCIM mouse ascites tumours by Klein and Révész may have appeared in the Rous tumour if host survival had been longer.

## SUMMARY AND CONCLUSIONS.

1. Ascites fluid from a rapidly growing Rous No. 1 ascites tumour was titrated intraperitoneally in 5-week-old brown leghorn chickens. As few as 100 tumour cells were sufficient to establish the ascites tumour but cell-free virus failed to do so. This adds further strength to the view that the final tumour cells are direct descendants of those initially inoculated.

2. A growth curve of the free tumour cells was constructed by determining the intraperitoneal free tumour cells in chickens at various intervals after the inoculation of a standard dose of $5 \times 10^6$ Rous cells. After an initial drop during the first 24 hours due to host response by phagocytosis and aggregation of cells at the surfaces of the peritoneum and pancreas, the log of the increase in free tumour cells was linear with time until 13 days after the inoculation. By 13 days all but one of the chickens were dead.

3. The maximum number of tumour cells reached was approximately $2{\cdot}5 \times 10^9$. The mean generation time for the tumour cells was 31 hours. The mitotic index of the free tumour cells rose from 2·00 to a maximum of 3·39 on the 10th day and fell on the 13th day to 1·50. Using the calculated mean generation time and observed mitotic indexes, an average mitotic time of 74 minutes was obtained.

4. Attachment of inoculated tumour cells to the host tissues and subsequent invasion was rapid. After 24 hours, aggregations of tumour cells were evident on the irregular surface of the pancreas and on the peritoneum. By 6 days invasion was widespread involving the peritoneum, pancreas, duodenum, spleen and ovary. It is concluded that the bird's early death is probably due to the extensive damage resulting from this invasion.

5. A period of about 4 days was required for the percentage of tumour cells in the total recoverable cells to rise to that prevailing at 0 time. After this, the percentage of tumour cells remained at about 50 per cent until the end of the tumour growth, and never reached the "nearly pure culture" stage of the mouse ascites tumours.

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## REFERENCES.

BATHER, R.—(1954) *Brit. J. Cancer*, **8**., 132.
CARR, J. G., AND HARRIS, R. J. C.—(1951) *Ibid.*, **5**, 83.
FRÄNKEL, E.—(1927) *Z. Krebsforsch.*, **25**, 407.
HUGHES, A.—(1952) 'The Mitotic Cycle'. London (Butterworth), p. 89.
KLEIN, G., AND RÉVÉSZ, L.—(1953) *J. nat. Cancer Inst.*, **14**, 229.
LASNITZKI, I.—(1952) *Brit. J. Cancer*, **7**, 238.
TENENBAUM, E.—(1943) *Brit. J. exp. Path.*, **24**, 56.

# INFLUENCE OF TUMOUR GROWTH RATE AND HOST AGE ON THE INFECTIVE VIRUS AND CYTOPLASMIC PARTICULATE YIELDS FROM SOME AVIAN TUMOURS.

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In a previous paper (Bather, 1953) it was shown that although the infectivity of purified Rous sarcoma virus preparations varied from 0–$10^7$ minimum infective doses (M.I.D.s) per g. tumour, the amount of purified virus material obtainable from the tumours remained fairly constant. The observations of Carr (1953*a*) and Duran-Reynals and Freire (1953) that host age and tumour growth rate influenced the infectivity of the virus were confirmed and used to control, in some degree, the production of virus of varying infectivities. The methods used for Rous sarcoma have now been extended to 31 Fujinami myxosarcomata and to a small number of $MH_2$ endotheliomata.

In addition, estimations have been made of the amounts of particulate material having similar chemical and physical properties to a virus but lacking infectivity, obtained from the non-filterable chemically-induced GRCH/16 sarcoma (Peacock and Peacock, 1953).

## MATERIALS AND METHODS.

All the chicks and chickens used in this work were obtained from the Intensity × Breeding line of the inbred flock of Brown Leghorns maintained at this centre.

The method for purifying preparations of Rous, Fujinami and $MH_2$ viruses and GRCH/16 particulates (using fractional centrifugation and enzyme treatment) the day-old chick method for titrating activity, and the biuret method for estimating the particles quantitatively have all been described in detail in previous papers (Carr and Harris, 1951 ; Bather, 1953). The estimates of the cell content of the tumour tissues were made by controlled homogenization in 0·88 M sucrose and 6 per cent acetic acid containing 0·0023 M $CaCl_2$ followed by dilution and counting in a haemacytometer (Mizen and Petermann, 1952 ; Bather, 1954). When working with the Fujinami tumour, it was found necessary to add 1 mg. hyaluronidase to the first homogenization mixture in order to break down most of the hyaluronate present which tended to coagulate in the acetic acid.

## EXPERIMENTAL.

### (1) *Tests of the ability of blood plasma from adult birds and birds bearing slow growing tumours to neutralize purified virus preparations.*

The development of naturally occurring antibodies in certain ageing fowl is well known (Duran-Reynals and Estrada, 1940). The flock maintained at Edinburgh has always been remarkably free from neoplastic diseases and the adult

chickens do not seem to acquire circulating antibodies to these conditions to any significant extent. It was thought, however, that it might be as well to test some of the adult birds and those bearing slow growing tumours to see if the blood of these fowls was capable of neutralizing virus infectivity. Such neutralization might account for the lowered infectivity of the virus obtained from tumours growing in these birds.

In all, the plasmas of 14 birds were tested, 12 from adult birds and 2 from birds bearing slow growing tumours. In the latter 2 cases, the slow growing tumours were also titrated for infectivity. Of the 12 adult birds tested, 4 were tested against Rous virus and 8 against Fujinami virus. Of the birds bearing slow growing tumours, 1 was tested against Rous and 1 against Fujinami, each bird bearing a corresponding type of slow tumour. The adult birds tested were between 1 and 3 years of age.

In order to simulate the conditions prevailing during a routine purification procedure, the plasma under test was added to the tumour tissue during the homogenization in water. The test plasma was thus in contact with the virus for just as long as the host antibodies would be. Since a large excess of test plasma was added (between 2–4 ml. to 4 g. tumour), any antibody in the test plasmas would have a much greater chance of exerting its inhibitory effect on the virus than it would under normal conditions.

The tumours, both control and with added test plasma, were processed in the usual way to obtain purified virus preparations. Table I summarizes the results of the infectivity titres performed by the day-old chick method already described. Infectivity is expressed as minimum infective chick doses (M.I.D.) per g. of fresh tumour.

Obviously the addition of the suspected plasmas to the tumours had little or no effect on the final activity of the purified extracts. Either adult birds and those bearing slow growing tumours contained no neutralizing antibodies or

TABLE I.—*Absence of Circulating Antibody Inhibition of Purified Rous or Fujinami Virus.*

| Tumours and plasmas tested. | Infective titre M.I.D.s/g. Untreated tumour control | Tumour + plasma. | Slow growing tumour. |
|---|---|---|---|
| Rous + plasma from adult bird | $10^3$ | $10^3$ | — |
| " " " " " | $10^2$ | $10^2$ | — |
| " " " " " | $10^3$ | $10^3$ | — |
| " " " " " | $10^3$ | $10^3$ | — |
| Rous + plasma from bird with slow growing Rous tumour | $10^3$ | $10^3$ | 0 |
| Fujinami + plasma from adult bird | $10^5$ | $10^4$ | — |
| " " " " " | $10^4$ | $10^5$ | — |
| " " " " " | $10^5$ | $10^5$ | — |
| " " " " " | $10^5$ | $10^6$ | — |
| " " " " " | $10^4$ | $10^5$ | — |
| " " " " " | $10^4$ | $10^4$ | — |
| " " " " " | $10^4$ | $10^5$ | — |
| " " " " " | $10^4$ | $10^4$ | — |
| Fujinami + plasma from bird with slow growing Fujinami tumour | $10^3$ | $10^3$ | $10^4$ |

the purification procedure removed them. Claude (1937) showed that the virus of Rous No. 1 sarcoma could be separated from antibody by high speed centrifugation, while trypsin treatment also increases the activity of virus preparations, probably by attacking antibody. It is more likely, however, that the natural incidence of circulating antibody is low in this flock.

(2) *Purified virus yields and infectivity titrations of slow and fast growing Fujinami myxosarcomata in young and adult hosts.*

Two groups of birds were used in this experiment, a young group (6 weeks), of the same hatch and an adult group (73–78 weeks). The birds in each group were all inoculated at the same time in each pectoral muscle with 0·5 ml. of a 10 per cent saline cell suspension of tumour, and sacrificed when the tumours were 18 days old in the case of the young group and 23 days for the adult group. Tumour size was determined by the + sign method, + being just palpable, ++ covering one quarter, +++ one half and ++++ the entire pectoral region.

After removal from the birds, the tumours were stored in Petrie dishes in the deep freeze. The virus retains its activity for considerable periods under these conditions (Epstein, 1951). Some of the tumours were processed immediately and the remainder within 2 weeks.

The results of the infectivity titrations and purified virus yields are summarized in Table II and III.

TABLE II.—*Infectivity of 31 Purified Extracts of Slow and Fast Growing Fujinami Myxosarcomata from Adult and Young Hosts.*

| | Infectivity M.I.D.s/g. tumour. | | | | | |
|---|---|---|---|---|---|---|
| | $0–10^3$ | | $10^3–10^4$ | | $10^5–10^6$ | |
| Host Age Group. | Slow. | Fast. | Slow. | Fast. | Slow. | Fast. |
| Young (6 weeks) | 2 | 2 | 1 | 4 | — | 6 |
| Adult (73–78 weeks) | 5 | 1 | 3 | 6 | — | 1 |

TABLE III.—*Average Virus Concentrate Yields Arranged in Order of Increasing Virus Infectivity.*

| Infectivity M.I.D.s/g. tumour. | Number of observations. | Mean purified virus yield and S.E. |
|---|---|---|
| $0–10^3$ | 10 | $0·39 \pm 0·04$ |
| $10^3–10^4$ | 14 | $0·38 \pm 0·03$ |
| $10^5–10^6$ | 7 | $0·32 \pm 0·05$ |

Total average yield $\pm$ standard error $= 0·37 \pm 0·03$.

TABLE IV.—*Analysis of Variance of the Average Virus Concentrate Yields from Fujinami Tumours Grouped According to Increasing Yield.*

| Source of variation. | Sum of squares. | Degrees of freedom. | Mean square. |
|---|---|---|---|
| Between groups (Table III) | 0·0181 | 2 | 0·00905 |
| Within groups (error) (Table III) | 0·4892 | 28 | 0·01747 |

From the Tables II and III it is apparent that Fujinami virus, like Rous virus, produces more fast-growing tumours exhibiting high infectivity in young birds than it does in adult hosts. Only 1 out of 16 adult birds grew a tumour of $10^5$ M.I.D.s/g. as compared with 6 out of 15 young birds, one of which reached a titre of $10^6$ M.I.D.s/g. As with Rous, Fujinami virus from slow growing tumours was usually less infective than virus from fast growing ones.

The range of infectivities for the whole group was from 0 to $10^6$ M.I.D.s/g. Purified virus yields, however, remained fairly constant at 0·37 mg./g. tumour. When divided into groups of low, medium and high infectivity (Table III) no significant differences appeared in the virus yields. Analysis of variance of the data is given in Table IV from which it is again obvious that there is no significant variation between the means of the 3 groups. Significance would require that the mean square of variation between the groups be greater than the mean square of variation within the groups.

Fujinami, therefore, behaves in precisely the same way as Rous No. 1 sarcoma in these respects. The only difference is a small one on the yield of purified virus per g. of tumour.

(3) *Purified particulate yields from slow and fast growing GRCH/16 tumours in young and adult hosts.*

Two groups of birds were used in this experiment as in the previous one. The young group were 6 weeks old of the same hatch and the adult group were 64–69 weeks old. The birds of each group were inoculated with 0·5 ml. of a 10 per cent cell suspension in saline in each pectoral muscle. As this tumour is more slowly growing than either of the filterable sarcomas, the birds were allowed to grow their tumours for 6 weeks. By this time it was possible to divide them into groups in slow and fast growing types on the basis of the + sign method as before. However, the demarcation between the two types was not so clear as with the filterable tumours and it is likely that the division into groups using the criterion of size does not carry as much significance in this group as in the filterable tumours.

After removal from the birds, the tumours were either processed immediately or after storage in the deep freeze. Preliminary experiments showed that GRCH/16 tissue stored in the deep freeze at −25° C. for up to 1 month was capable of producing a tumour upon inoculation into chickens and all extractions were done within this time. Table V shows the mean purified particulate yields from the various groups together with their standard errors. The purifications and particulate estimations were done in the same way as before. As the tumour is not filterable, no infectivity titrations were possible.

TABLE V.—*Average Purified Cytoplasmic Particulate Yields from Slow and Fast Growing GRCH/16 Tumours in Young and Adult Hosts.*

| | Slow growing. | | Fast growing. | |
|---|---|---|---|---|
| Host age group. | Number of observations. | Mg/g. tumour (and S.E.). | Number of observations. | Mg/g. tumour (and S.E.). |
| Young (6 weeks) . . | 9 | 4·72±0·36 | 6 | 4·98±0·44 |
| Adult (64–69 weeks) . . | 5 | 4·12±0·49 | 12 | 4·66±0·31 |

Total average yield ± standard error = 4·65 ± 0·18.

TABLE VI.—*Analysis of Variance of the Mean Particulate Yields from the Groups of GRCH/16 Tumours given in Table V.*

| Source of variation. | Degrees of freedom. | Sum of squares. | Mean squares. |
|---|---|---|---|
| Between age groups | 1 | 0·808 | 0·808 |
| Between growth rate groups | 1 | 0·568 | 0·568 |
| Interaction | 1 | 0·742 | 0·742 |
| Within groups (error) | 28 | 33·031 | 1·179 |

Again, little variation in the yields of particulates, similar in size and chemical composition to Rous virus but lacking infectivity, was found in any of the groups. The yield per g., however, was much higher and bears out Howatson's observations (personal communication) using the electron microscope. Analysis of variance of the data in Table V is given in Table VI for the 2-way classification —age of host and rate of growth. Again all main effects and their interaction are insignificant.

(4) *Purified virus yields and infectivities of some $MH_2$ endotheliomata.*

Some difficulty has been experienced in maintaining the $MH_2$ endothelioma and consequently the results so far include only 8 tumours grown in young (6-week old) chickens. The left thigh was used as the site of injection of 0·5 ml. of a 10 per cent cell suspension in saline. The birds were sacrificed 26 days later and arbitrarily divided into two groups of 4 birds each in the basis of tumour size. The smallest tumours were called slow growing and the largest, fast growing. Infectivity titrations and virus estimations were done on purified extracts as before. The 8 results are summarized in Table VII. Bird No. 4 of the slow growing group bore a very small tumour yielding too little purified virus to be measured by the biuret technique.

TABLE VII.—*Infectivity and Purified Virus Content of 4 Slow Growing and 4 Fast Growing $MH_2$ Endotheliomata.*

| Bird No. | Tumour growth rate. | Virus content. M.I.D.s per g. | Virus content. Mg. dry weight per g. |
|---|---|---|---|
| 1 | Slow | $10^2$ | 0·71 |
| 2 | ,, | $10^2$ | 0·22 |
| 3 | ,, | $10^3$ | 1·39 |
| 4 | ,, | $10^3$ | — |
| 1 | Fast | $10^3$ | 0·06 |
| 2 | ,, | $10^4$ | 0·58 |
| 3 | ,, | $10^5$ | 0·23 |
| 4 | ,, | $10^5$ | 0·95 |

Average ± standard error = 0·59 ± 0·18.

Although the results are few, the trend is obviously the same as that present in the other two filterable tumours. Here, a range of 4 log doses is available but the yields of purified virus, though rather variable, bear no relationship to the

infectivity. If the animals are divided into groups of low and high infectivity the low group ($10^2$ and $10^3$ M.I.D.s per g.) yield an average of 0·60 mg./g. while the high group ($10^4$ and $10^5$ M.I.D.s per g.) yield an average of 0·59 mg. /g. The total average yield is 0·59 mg./g. As with the Rous and Fujinami tumours, the slow growing $MH_2$ tumours contain less *infective* virus than the fast growing ones with one exception. Unfortunately, the author was unable to obtain any data for tumours grown in adult birds owing to difficulties of transmission.

(5) *Purified virus and particulate yields expressed as weight per cell.*

It was thought that a more accurate idea of the true virus and particulate yields from the tumours studied might be gained by expressing them as dry weight per cell. Cell counts were done, therefore, by the homogenization methods described. In Table VIII the values obtained for the 4 tumours are presented along with the similar values worked out from stained microscopic slides. The slides were made by fixed small pieces of tumour in Susa and staining with haematoxylin and eosin. Thirty different fields were examined and cells counted with the aid of crossed hairs in the eyepiece. By calibrating the diameter of the field, the average number of cells for a depth of $5\mu$ (the average depth of field) was obtained. Knowing the area of the field the cell count per g. could then be calculated.

TABLE VIII.—*Average Cell Content of Rous, Fujinami, $MH_2$ and GRCH/16 Tumours Estimated by the Homogenization Procedure and by Direct Counting of Stained Slides.*

| Tumour. | Number of observations. | Cell content per g. tissue. Homogenization (± S.D.) | Direct count. |
|---|---|---|---|
| Rous* | 5 | $32·2 \pm 2·9 \times 10^7$ | $28·8 \times 10^7$ |
| Fujinami | 7 | $29·0 \pm 2·1 \times 10^7$ | $27·5 \times 10^7$ |
| $MH_2$ | 4 | $50·8 \pm 1·3 \times 10^7$ | $52·2 \times 10^7$ |
| GRCH/16 | 4 | $87·2 \pm 3·0 \times 10^7$ | $86·4 \times 10^7$ |

* From Bather (1954).

TABLE IX.—*Virus and Particulate Yields Recalculated as Dry Weight per Cell and Numbers of Particles per Cell.*

| Tumour. | Average virus or particulate yield. Mg. per cell. | Number of particles per cell. |
|---|---|---|
| Rous* | $1·43 \times 10^{-9}$ | 4,500 |
| Fujinami | $1·28 \times 10^{-9}$ | 4,000 |
| $MH_2$ | $1·16 \times 10^{-9}$ | 3,600 |
| GRCH/16 | $5·33 \times 10^{-9}$ | 16,600 |

* From Bather (1954).

The close agreement by the two methods is apparent and the standard deviations of the homogenized preparations gives an indication of the reproducibility of the results by this method. Samples of tumour from both slow and fast growing types were included and the cell counts were always very similar.

It has now become possible to recalculate the virus and particulate yields

as dry weight per cell and this has been done in Table IX using the virus yields from the previous experiments. The final column gives an approximation to the number of virus or cytoplasmic particulates assuming a mass of $3{\cdot}2 \times 10^{-16}$ g. for each particle (calculated for a sphere of radius $4{\cdot}0 \times 10^{-6}$ cm. with a density of 1·3—values calculated for Rous sarcoma virus from centrifuge, filtration and electron microscope studies). It was felt that a comparison in this way was justifiable since the size range of the particles selected by a given procedure is dependent on such things as the force of the centrifugal field applied and the degree of homogenization. In all the extractions done throughout this work, the same conditions have applied, and it is almost certain that the purified concentrates are made up of particles within the same size range. Howatson (1953) has made this clear by photographing a wide variety of tissue extracts made in the same way, in the electron microscope. Photographs of $MH_2$ (filterable) tumour virus and GRCH/16 particulates show a very similar size range.

On this basis, the apparent differences between the yields from each tumour are considerably reduced. It is now seen that the three filterable sarcomata contain roughly similar amounts of purified virus material, whereas the non-filterable GRCH/16 tumour contains almost exactly 4 times as much particulate material.

## DISCUSSION.

The results presented in this paper extend to two other virus induced tumours a relationship already found in Rous No. 1 sarcoma. That is, that despite the wide variation in infective power of each tumour, each contains a fairly constant amount of virus material which can be extracted, purified by fractional centrifugation and enzyme treatment and estimated by a modified biuret reaction. When converted to numbers of elementary particles per cell (Table VIII) it is seen that each of the three filterable tumours investigated yield approximately similar amounts of purified virus. The non-filterable GRCH/16 tumour cells on the other hand, yielded roughly 4 times as many particles similar in size, density and chemical composition to the viruses from the filterable tumours. Obviously the property of infectivity does not depend solely on the production of these particles. Why the GRCH/16 tumour should produce so much particulate material of this general size and chemical composition is not clear at the moment. However, it is interesting in this connexion to note that there is a slight, though insignificant, inverse relationship between infectivity and amount of purified virus material obtainable from both Rous (Bather, 1953) and Fujinami tumours. There are not enough results available for the $MH_2$ endothelioma to see if this relationship holds for all the filterable tumours studied. Recently Albert and Johnson (1954) discovered that primary azo dye-induced cholangiomata contained about $\frac{1}{2}$ and transplanted hepatoma cells about $\frac{1}{5}$ as many cytoplasmic particles as normal liver. The relative amounts of particles were estimated on the basis of their nitrogen content. These results together with the high yield of particles from GRCH/16 (which is slower growing than either of the filterable tumours) suggests that it may be worth while investigating further the possible influence of growth rate and metabolism on the "small granule" fraction of cytoplasm.

In the previous paper, the implications of the results for Rous sarcoma were discussed. It was suggested that the slow growing tumours and those growing in old birds were unable to complete the formation of infective virus to the same

extent as fast growing and tumours in young hosts could. This would result in a lower proportion of " complete " infective virus in the former, assuming the life cycle of the virus to resemble that of other viruses (Carr, 1953*b*). The production of incomplete, non-infective virus is known to occur frequently in the plant, animal and bacterial virus fields and its appearance can, to some extent, be controlled (Bawden and Pirie, 1946 ; Gard and von Magnus, 1946 ; Doermann, 1951 ; Fazekas de St. Groth and Graham, 1954*a*, 1954*b*). The uniqueness of the tumour viruses lies in their failure to be released from the cells after the first growth step, the newly formed agents being distributed to new cells by cell division instead of reinfection. The property of infectivity appears to be redundant in tumour viruses unless one admits that it may be of use under natural conditions in its transmission by insect vectors (Carr, 1953*c* ; Johnston, 1937). That low infectivity was not due to neutralization by circulating antibodies is clear from the results in Table I.

The yields of cytoplasmic particulates from the non-filterable GRCH/16 tumour were not significantly different when isolated from slow or fast growing tumours in old or young hosts. The question arises whether or not these particulates are concerned with the growth of the tumour. Claude (1940, 1943) found particles in a variety of normal cells which corresponded in size and chemical composition to the avian tumour viruses, and the electron microscope showed such particles to exist in the cytoplasm of normal fowl macrophages and the cells of the pancreas and salivary glands. In the latter cells they appeared as filaments or sheets (Dalton, Kahler, Streibich and Lloyd, 1950 ; Bernhard, Gautier and Oberling, 1951) or in the form of tubes (Palade, 1952). Their wide variation in quantity and variations in size, properties shared with the viruses of avian sarcomas (also shown by electron microscope studies), suggests that these particles are capable of multiplication. A recent electron microscope study of similar inclusions in rat hepatoma cells (Oberling, Bernhard, Gautier and Hagenau, 1953) shows that they, too, present very wide variations in the numbers of particles in the cytoplasm. Although no such data is yet available for the GRCH/16 tumour, there is every reason to believe that it would present the same picture, and that the vigorous multiplication of particles of this size range is a common property of cells undergoing a high rate of metabolism and protein synthesis. Whether in the case of tumour cells they are akin to viruses (non-infective) is another matter and one which must remain in the field of theory until such time as proof of their genetical continuity is forthcoming. Kidd (1946) presented what might be called evidence in favour of virus-like qualities of particulates from a non-filterable tumour, the Brown-Pearce tumour of rabbits. He obtained a high molecular weight protein which gave a specific complement fixation reaction with the sera of rabbits previously injected with cells or tumour extracts. Treatment of cells of the Brown-Pearce tumour with antiserum made from its extracts, resulted in either feeble growth or none at all when inoculated into rabbits. Similar results have been claimed for the mouse lymphosarcoma 6C3HED by Nungester and Fisher (1954). These findings parallel some of the immunological experiments done with virus induced tumours, and the results suggest the possibility of a " masked " or " incomplete " type of agent.

SUMMARY.

Purified virus yields from 31 Fujinami myxosarcomata and 8 $MH_2$ endotheliomata were estimated by the biuret colorimetric procedure. No significant

relationship was found to occur between infectivity (day-old chick titration) and the amount of virus obtainable.

The results for Fujinami parallel those found earlier for Rous No. 1 sarcoma in that virus from fast growing tumours and tumours from young hosts was more infective than that from slow growing tumours and from tumours in adult hosts. Virus from fast growing $MH_2$ tumours also appeared to be more infective than that from slow growing ones. The reduction in infectivity was not due to neutralization by circulating antibodies in adult hosts or those bearing slow tumours.

Cytoplasmic particulate yields from the non-filterable GRCH/16 sarcoma were not significantly different whether isolated from fast or slow growing tumours or young or adult hosts.

When recalculated as average number of particles per cell, the virus and particulate yields became: Rous, 4,500; Fujinami, 4,000; $MH_2$, 3,600; GRCH/16–16,600 particles per cell.

The implications of these results are discussed and it is concluded that all the virus induced tumours so far studied exhibit alterations in the ratio infective : non-infective virus which depend on the rate of growth of the tumour and age of the host. The faster the tumour growth rate and the younger the host, the more complete, infective virus produced.

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## REFERENCES.

ALBERT, S., AND JOHNSON, R. M.—(1954) *Cancer Res.*, **14**, 271.
BATHER, R.—(1953) *Brit. J. Cancer*, **7**, 492.—(1954) *Ibid.*, **8**, 132.
BAWDEN, F. C., AND PIRIE, N. W.—(1946) *Brit. J. exp. Path.*, **27**, 81.
BERNHARD, W., GAUTIER, A., AND OBERLING, CH.—(1951) *C. R. Soc. Biol., Paris*, **154**, 556.
CARR, J. G.—(1953*a*) 'The Nature of Virus Multiplication.' Oxford (Cambridge University Press), p. 287.—(1953*b*) *Proc. Roy. Soc. Edinb.*, **65**, Part 1 (No. 5), 66.—(1953*c*) *Experientia*, **9**, 326.
*Idem* AND HARRIS, R. J. C.—(1951) *Brit. J. Cancer*, **5**, 83.
CLAUDE, A.—(1937) *J. exp. Med.*, **66**, 59.—(1940) *Science*, **91**, 77.—(1943) *Ibid.*, **97**, 451.
DALTON, A. J., KAHLER, H., STRIEBICH, M. J., AND LLOYD, B.—(1950) *J. nat. Cancer Inst.*, **11**, 439.
DOERMANN, A. H.—(1951) *Fed. Proc.*, **10**, 591.
DURAN-REYNALS, F., AND ESTRADA, E.—(1940) *Proc. Soc. exp. Biol., N.Y.*, **45**, 367.
*Idem* AND FREIRE, P. M.—(1953) *Cancer Res.*, **13**, 376.
EPSTEIN, M. A.—(1951) *Brit. J. Cancer*, **5**, 317.
FAZEKAS DE ST. GROTH, S., AND GRAHAM, DORIS, M.—(1954*a*) *Brit. J. exp. Path.*, **35**, 60.—(1954*b*) *Nature*, **173**, 637.
GARD, S., AND VON MAGNUS, P.—(1946) *Ark. Kemi. Min. Geol.*, **24B**, No. 8.
HOWATSON, A. F.—(1953) *Brit. J. Cancer*, **7**, 393.
JOHNSTON, E. P.—(1937) *Poult. Sci.*, **16**, 225.
KIDD, J. G.—(1946) *J. exp. Med.*, **83**, 227.
MIZEN, NANCY A., AND PETERMAN, MARY L.—(1952) *Cancer Res.*, **12**, 727.
NUNGESTER, W. J., AND FISHER, HELEN.—(1954) *Ibid.*, **14**, 284.
OBERLING, CH., BERNHARD, W., GAUTIER, A., AND HAGUENAU, FRANÇOISE.—(1953) *Pr. méd.*, **61**, 719.
PALADE, G. E.—(1952) *J. exp. Med.*, **95**, 285.
PEACOCK, P. R., AND PEACOCK, ANDREE.—(1953) *Brit. J. Cancer*, **7**, 120.

# THE INTRACELLULAR METABOLISM OF 3 : 4 BENZPYRENE: SITES OF OXIDATION IN MOUSE LIVER.

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STUDIES of the mechanism of action of the polycyclic hydrocarbons within the animal body have been mainly concerned with gross effects. Specific action within particular tissues has only been touched upon, whilst action within the individual cells of tissues has been barely considered. An understanding of the mechanism of carcinogenesis must involve a knowledge of the intracellular behaviour of the carcinogenic agent. Benzpyrene is a very suitable agent for such studies as it can be traced by way of its fluorescence and absorption spectra. Additionally, certain information is available as to the nature and properties of the primary metabolic derivatives.

Founded upon these considerations we have attempted a detailed investigation of the action of 3 : 4 benzpyrene within cells, both from the aspect of metabolic change of the hydrocarbon itself and from the viewpoint of effects upon cellular constituents. Throughout the work we have endeavoured to consider the problem in relation to the cell structure as any interpretation of biological behaviour must be couched in terms of cell morphology to be intelligible.

The present paper details the results obtained in studies of the sites at which benzpyrene is oxidised within mouse liver. A preliminary account of this work has appeared, (Calcutt and Payne, 1954).

*Present knowledge of benzpyrene metabolism.*

The starting point for the present series of investigations is the data collected by Weigert and Mottram (1946*a*, 1946*b*). These authors reviewed the existing knowledge and then gave a detailed account of experiments designed to trace the primary steps in the metabolism of benzpyrene in whole tissues. They isolated and characterised two compounds as primary metabolites which were labelled as $BpX_1$ and $BpX_2$. By physical methods and analogy with known derivatives of other polycyclic hydrocarbons these were shown to be :

8 ($OR_1$) – 9 (OH) – 8, 9 dihydro – 3, 4 benzpyrene ($BpX_1$) and
8 ($OR_1$) – 9 ($OR_2$) – 8,9 dihydro– 3, 4 benzpyrene ($BpX_2$).

The radicals $R_1$ and $R_2$ are unknown.

These two compounds were formed in the tissues where benzpyrene was metabolised and then converted to the final excretion products, 8. OH benzpyrene and 5 : 8 benzpyrene quinone, during their passage through the animal body. A further compound labelled $BpF_1$ and believed to be 8 ($OR_1$)—3, 4 benzpyrene, was found to occur as an intermediate stage in the dehydration of $BpX_1$ to 8.OH

benzpyrene. In the course of this work it was found that the benzpyrene derivatives present in a tissue at any particular time were firmly bound to tissue constituents and could only be separated after treatment with acetone.

More recently, Miller (1951) has found that benzpyrene applied to mouse skin becomes firmly bound to a protein fraction. Using fluorescence spectroscopy as the detecting method Calcutt and Payne (1953) found benzpyrene to persist in the nuclei and mitochondria of mouse liver for long periods after intraperitoneal injection of the carcinogen. This parallels the finding by Wiest and Heidelburger (1953) that 1 : 2 : 5 : 6 dibenzanthracene is bound in the nuclei, mitochondria, microsomes and supernatant of submaxillary glands of mice after local application of the hydrocarbon.

The work summarised above forms the foundation of our present researches.

*Materials used.*

Young mice of either the Strong A or R III strains were used in all experiments. Early experience showed that there was no detectable strain differences in metabolism so mice were used as available. The liver was chosen for first experiments as being easily handled, of adequate bulk, and as a site where metabolism of benzpyrene is known to occur. The benzpyrene was from a stock held in this laboratory and giving an absorption spectrum identical with those in the literature for the pure hydrocarbon.

The ultimate aim was the detection of metabolites by spectroscopic methods, so all reagents used were selected as being of "Analytical Reagent" quality. Organic solvents were either purchased as suitable for spectroscopic work or further purified to remove interfering contaminants. Silica for chromatography was prepared by the method of Gordon, Martin and Synge (1943); the fraction of 100/150 mesh being used. Alumina was a commercially available product.

*Preliminary experiments.*

Since benzpyrene has been shown to be concentrated in nuclei and mitochondria attempts were made to determine whether metabolism occurs at these sites. At intervals after the intraperitoneal injection of 0·1 mg. of benzpyrene into mice the animals were killed, the livers removed and the nuclei and mitrochondria extracted in 0·88 M sucrose solution. Extracts of these fractions were made with various solvents including ethyl alcohol, ethyl ether, amyl alcohol, benzene and cyclohexane. Examination of the extracts in the spectrophotometer showed the presence of benzpyrene together with various unidentifiable substances derived from the tissue fraction. As it was likely that the spectra of any metabolites present were being obscured by other compounds, purification was attempted by chromatography on either alumina or silica columns. No metabolites were found, but it was discovered that a yellow pigmented substance could be extracted from both nuclei and mitochondria. This could not be separated from benzpyrene on alumina columns, but obviously had no connection with the problem since it could also be extracted from nuclei and mitrochondria from untreated control animals. Spectroscopically this substance showed a steady fall over the region 220–340 m$\mu$. and then two maxima at 367 and 387 m$\mu$. In the presence of benzpyrene it showed the superimposed maxima of the hydrocarbon at 265 m$\mu$.,

285 m$\mu$. and 296 m$\mu$. and ill-defined maxima spread over 365–367 m$\mu$. and 384–387 m$\mu$. The spectra obtained in this fashion closely resemble that found for a believed benzpyrene metabolite from rats by Reggiani, Dansi and Morelli (1939). As these authors prepared their material by extraction with benzene and chromatography on alumina we are of the opinion that their finding was of a mixture of benzpyrene and an unknown cell constituent and, like ours, cannot be a benzpyrene derivative.

Another series of experiments was undertaken in collaboration with Dr. R. Barer of the Department of Human Anatomy, Oxford. These were based on the finding by Barer, Ross and Tkacyzk (1953) that suspension of living particulate material in a medium of the same refractive index as the material itself will, provided that leakage of the external medium into the particles does not occur, produce a suspension which is virtually transparent. Mitochondria were extracted from normal and benzpyrene-treated livers of rats and mice. On immersion in suitable solutions of purified bovine plasma albumin and examination by phase contrast microscopy it was found that phase reversal occurred at protein concentrations equivalent to 50–52 per cent solid matter. No distinction was detected between the mitochondria from normal livers and those from the hydrocarbon treated tissue. Thick suspensions of mitochondria were made in appropriate protein solutions and examined in the spectrophotometer; protein solution being used in the control cell. The spectra obtained were useless for our purposes as the absorption by normal constituents of the mitochondria completely masked any due to the hydrocarbon or its derivatives. These experiments did, however, demonstrate that freshly prepared mitochondria are impervious to albumin solutions. This suggests that the mitochondria membranes demonstrated by Sjöstrand (1953) should be regarded as semi-permeable.

Ultimately we adopted a method for the extraction and purification of metabolites founded upon that devised by Weigert and Mottram (1946*a*).

*Separation of cell fractions and isolation of benzpyrene metabolites.*

Batches of 6–8 mice were injected intravenously via the tail vein with 0·5 c.c. of colloidal benzpyrene in water, the concentration being such that each mouse received approximately 0·05 mg. of the hydrocarbon. This route of administration was adopted as being the one best calculated to give a concentration of hydrocarbon in the liver. At various preselected intervals after the injection the mice were killed, the livers removed and homogenised in Tyrode solution. This latter was selected as the homogenising medium as it was desired to maintain the pH above 7·0 since conversion of $BpX_1$ and $BpX_2$ to 8.OH benzpyrenol occurs under acid conditions. The liver suspension was strained through four layers of gauze to remove fibrous tissue and then spun down at 1,500 $\times$ g. for 15 minutes to sediment the nuclei. Mitochondria were collected after spinning for 20 minutes at 22,000 $\times$ g. in the Spinco Ultracentrifuge, and the microsomes were removed by a final spinning at 25,000 $\times$ g. for 30 minutes. The nuclear and mitochondrial fractions were each resuspended in Tyrode and spun down again to free them of contaminant material.

Acetone was then poured into each of the four fractions: nuclei, mitochondria, microsomes and supernatant. After a few minutes the acetone was removed by boiling off under reduced pressure and the fractions were repeatedly extracted with individual small portions of xylene. This was repeated until no further

fluorescent material was extracted. The xylene samples from each fraction were pooled together, dried over anhydrous sodium sulphate and passed through a chromatograph column comprised of silica layered over alumina. It had been found previously by Weigert and Mottram (1946*a*) that under these conditions $BpX_1$ and $BpX_2$ are adsorbed at the surface of the silica, the phenolic derivatives $BpF_1$ and 8.OH benzpyrene are adsorbed on the alumina and unchanged benzpyrene appears in the eluate.

After suitable washing with pure xylene the columns were extruded and cut into portions as determined by the positions of adsorbed fluorescent material. The individual portions were eluted with ethyl alcohol and then examined in the Unicam Spectrophotometer ; ethyl alcohol being used in the control cell. Spectra were taken over the range 340–430 m$\mu$. and compared with those published by Weigert and Mottram (1946*a*). In most cases one or more further chromatographic purifications were necessary before the spectra could be considered as representative of the compounds under investigation. Even under these circumstances the spectra were poor as they were derived from very small amounts of material and often still showed indications of contaminants. Confirmation of the nature of any one sample as determined by absorption spectroscopy was sought in its behaviour on the chromatograph columns and its fluorescence spectrum. Determinations of metabolites as detailed below are then based on a combination of absorption spectra, fluorescence spectra and chromatographic behaviour.

*Experimental findings.*

The experiments in this series were only carried on over a period of up to 24 hours, since the bulk of benzpyrene deposited in the liver after intravenous injection is excreted within this time period. The general results are detailed in Table I.

TABLE I.—*Benzpyrene and Derivatives in Mouse Liver Fractions after Intravenous Injection of the Colloidal Hydrocarbon.*

| Number of mice used. | Time between injection and killing (hours). | Extraction medium. | Findings. | | | |
|---|---|---|---|---|---|---|
| | | | Nuclei. | Mitochondria. | Microsomes. | Supernatant. |
| 6 | 2½ | Tyrode (pH—7·6) | Bp ; $BpX_2$ | Bp ; $BpX_2$ | Bp<br>$BpX_2$ | Bp (trace)<br>$BpX_1$ ; $BpX_2$ |
| 8 | 2½ | Tyrode (pH—7·6) | — | — | Bp ; $BpX_2$ | $BpX_1$ ; $BpX_2$ |
| 7 | 4 | Tyrode (pH—7·6) | Bp ; $BpX_2$ | Bp ; $BpX_2$ | Bp ; $BpX_2$ | Bp (Trace)<br>$BpX_1$ ; $BpX_2$ |
| 7 | 8 | Tyrode (pH—7·6) | Bp ; $BpX_2$ | Bp ; $BpX_2$ | Bp ; $BpX_2$ | $BpX_2$ |
| 5 | 12 | Sucrose 0·88 M (pH—6·8) | — | Bp ; $BpX_2$ | $BpX_2$ | Bp<br>$BpX_1$ ; $BpX_2$ |
| 5 | 17 | Tyrode (pH—7·6) | Bp ; $BpX_2$ | Bp ; $BpX_2$ | $BpX_2$ | Bp<br>$BpX_1$ ; $BpX_2$ |
| 7 | 17 | 1 per cent citric acid (pH—2·6) | Bp ; $BpX_2$ | Bp ; $BpX_2$ | $BpX_2$ | $BpX_1$ ; $BpX_2$<br>$BpF_1$ ; $BpF_2$ |
| 8 | 17½ | Tyrode (pH—7·6) | Bp ; $BpX_2$ | $BpX_2$ | $BpX_2$ | $BpX_1$ |
| 5 | 24 | Tyrode (pH—7·6) | Bp ; $BpX_2$ | Bp ; $BpX_2$ | Bp ; $BpX_2$ | Bp (Trace)<br>$BpX_1$ ; $BpX_2$ |

Abbreviations used : Bp. = 3 : 4 benzpyrene.
$BpX_1$ = 8 ($OR_1$) – 9(OH) – 8,9-dihydro 3 : 4 benzpyrene.
$BpX_2$ = 8 ($OR_1$) – 9($OR_2$) – 8,9-dihydro 3 : 4 benzpyrene.
$BpF_1$ = 8 ($OR_1$) 3 : 4 benzpyrene.
$BpF_2$ = 8.OH 3 : 4 benzpyrene.
Blank space means that the particular fraction was not examined.

In carrying out these experiments we were aware that saline solutions are perhaps not the best media for extraction of cell fractions. We have therefore checked our general results by some extractions in other media. Citric acid (1 per cent) was used according to the technique of Mirsky and Pollister (1946) as in our experience this gives the best yield of nuclei uncontaminated by other cell constituents. Sucrose (0·88 M) was also used as advocated by Hogeboom, Schneider and Pallade (1948) in order to achieve well preserved mitochondria of a high degree of purity. The results in both cases conformed to those obtained with Tyrode as the diluting agent. In the case of citric acid the supernatant fraction contained some of the phenolic derivatives $BpF_1$ and $BpF_2$. These probably arose as breakdown products of $BpX_1$ or $BpX_2$ during the extraction process.

The results may be summarised as :

*Nuclei.*—Unchanged benzpyrene and $BpX_2$ present throughout.

*Mitrochondria.*—Unchanged benzpyrene and $BpX_2$ present throughout.

*Microsomes.*—$BpX_2$ present throughout. Unchanged benzpyrene present in all samples up to 8 hours, but only one out of five from longer time intervals.

*Supernatant.*—$BpX_1$ and $BpX_2$ regularly occurred. Unchanged benzpyrene only found occasionally and then only in trace amounts.

It may be argued that the distribution of benzpyrene metabolites as found is not representative of intracellular behaviour but arises as a result of adsorption during the homogenising or centrifuging processes. To overcome this objection we have separated normal untreated livers into the same fractions as previously. These have been resuspended in colloidal suspensions of benzpyrene and then placed in the incubator at 37° C. for varying time intervals. Throughout this period they were maintained in complete darkness so as to avoid any photosensitising activity by the hydrocarbon. The fractions were then processed as previously and checked for metabolites. The results are given in Table II.

TABLE II.—*Benzpyrene Derivatives found after Incubation of Mouse Liver Fractions with Colloidal Benzpyrene.*

| Number of mice used. | Incubation time. (hours). | Extraction medium. | Incubation medium. | Findings. | | | |
|---|---|---|---|---|---|---|---|
| | | | | Nuclei. | Mitochondria. | Microsomes. | Supernatant. |
| 5 | 1½ | Tyrode (pH—7·6) | Tyrode (pH—7·6) | $BpX_2$ | $BpX_2$ | — | $BpX_1$ $BpX_2$ |
| 6 | 1½ | Sucrose—0·88M (pH—6·8) | Sucrose—0·25M (pH—6·8) | $BpX_2$ | $BpX_2$ | $BpX_2$ | $BpX_1$ |
| 6 | 2 | Tyrode (pH—7·6) | Tyrode (pH—7·6) | — | — | — | $BpX_1$ $BpX_2$ |
| 6 | 2½ | 1 per cent citric acid (pH—2·6) | Tyrode (pH—7·6) | $BpX_2$ | $BpF_2$ | $BpX_2$ $BpF_2$ | — |
| 8 | 16 | Tyrode (pH—7·6) | Tyrode (pH—7·6) | $BpX_2$ | $BpX_2$ | — | $BpX_1$ $BpX_2$ |

Abbreviations as in Table I.

Comparison of Tables I and II shows that the findings from *in vivo* experiments are completely substantiated by the *in vitro* work. In some cases phenolic derivatives were found in the material from the *in vitro* experiments, but this is not surprising in view of Weigert and Mottram's (1946*a*) finding that the primary derivatives are rapidly converted to phenolic compounds in the presence of tissues.

Throughout these experiments no evidence has arisen to suggest that any other benzpyrene metabolites are formed.

## DISCUSSION.

The results obtained in these experiments are consistent with previous findings and also with data obtained with other carcinogens. We have shown that benzpyrene becomes distributed throughout the cell, as was found for dibenzanthracene by Wiest and Heidelburger (1953). The hepatic carcinogen 4-dimethylaminoazobenzene has been found by Price, Miller and Miller (1948) and Price, Miller, Miller and Weber (1949) to occur in all four fractions of rat liver. The completely unrelated compound 2-acetylaminofluorene has been shown by Weisburger, Weisburger and Morris (1953) to extend to all cell fractions of rat liver and kidney. Danielli (1952) has also reported that nitrogen mustard penetrates throughout and has a "diffuse" action on the cell. If this ability to spread throughout the cell is found to be a general property of carcinogenic agents it may prove important and help to explain the multiplicity of apparently unrelated biological responses that these agents can elicit.

The biological implications of these findings cannot be adequately expressed till further experiments are undertaken, but several points are worthy of comment now. From their studies with whole tissues Weigert and Mottram (1946*b*) concluded that benzpyrene was initially oxidised to $BpX_1$ and then further changed to $BpX_2$ whilst still in contact with tissues. The present discovery that $BpX_1$ only occurs in the supernatant fraction is suggestive of its formation solely at this site and that $BpX_2$ is derived independently at alternative sites. This implies two independent mechanisms of oxidation with separate but related end products. Such a scheme also explains the fact that $BpX_1$ appears as an excretion product in the bile before $BpX_2$, whilst later $BpX_2$ predominates. Obviously, during its passage across the cell benzpyrene will come into contact with cytoplasmic material (extracted as supernatant) before it is absorbed by the particulate cell components. Assuming roughly equivalent rates of formation of the two derivatives it is clear that the relative amount of $BpX_1$ to $BpX_2$ will be higher in the initial period.

The widespread occurrence of benzpyrene metabolism within the cell would suggest that the oxidation processes are not specific enzyme mechanisms, since modern evidence indicates that enzymes tend to be localised. The alternative is that oxidation occurs as the result of interaction with some widespread normal cell constituent, perhaps structural protein. Such a view is supported by the findings of Calcutt (1949, 1950), Garzia and Dansi (1953) and Warren (1943) that benzpyrene can be oxidised in the presence of simple compounds (cysteine, ascorbic acid) which occur in cells, to give end products comparable to those found during metabolism in the animal.

## SUMMARY.

1. Pooled livers from groups of 6–8 mice which had been intravenously injected with colloidal benzpyrene were fractionated into nuclei, mitrochondria, microsomes and supernatant.

2. Examinations for benzpyrene metabolites at intervals up to 24 hours showed $BpX_2$ exclusively in nuclei, mitochondria and microsomes. $BpX_1$ and $BpX_2$ occurred in the supernatant fraction.

3. Corresponding fractions from untreated liver were incubated at 37° C. with colloidal benzpyrene. Metabolites were extracted as in the *in vivo* experiments, thus demonstrating that the oxidation of benzpyrene is at sites found.

4. The results and their significance are briefly discussed.

## REFERENCES.


BARER, R., ROSS, K. F. A. AND TKACYZK, S.—(1953) *Nature*, **171**, 270.

CALCUTT, G.—(1949) *Brit. J. Cancer*, **3**, 306.—(1950) *Ibid.*, **4**, 254.

*Idem* AND PAYNE, S.—(1953) *Ibid.*, **7**, 279.—(1954) *Nature*, **174**, 841.

DANIELLI, J. F.—(1952) *Ibid.*, **170**, 863.

GARZIA, A. AND DANSI, A.—(1953) *Farmaco*, **8**, 449.

GORDON, A. H., MARTIN, A. J. P. AND SYNGE, R. L. M.—(1943) *Biochem. J.*, **37**, 79.

HOGEBOOM, G. H., SCHNEIDER, W. C. AND PALLADE, G. E.—(1948) *J. biol. Chem.*, **172**, 619.

MILLER, E. C.—(1951) *Cancer Res.*, **11**, 100.

MIRSKY, A. E. AND POLLISTER, A. W.—(1946) *J. gen. Physiol.*, **30**, 117.

PRICE, J. M., MILLER, E. C. AND MILLER, J. A.—(1948) *J. biol. Chem.*, **173**, 345.

*Idem*, MILLER, J. A., MILLER, E. C. AND WEBER, G. M.—(1949) *Cancer Res.*, **9**, 96.

REGGIANI, M., DANSI, A. AND MORELLI, E.—(1939) *Tumori*, **25**, 635.

SJÖSTRAND, F. S.—(1953) *Nature*, **171**, 30.

WARREN, F. L.—(1943) *Biochem. J.*, **37**, 338.

WEIGERT, F. AND MOTTRAM, J. C.—(1946*a*) *Cancer Res.*, **6**, 97.—(1946*b*) *Ibid.*, **6**, 109.

WEISBURGER, E. K., WEISBURGER, J. H. AND MORRIS, H. P.—(1953) *Arch. Biochem. Biophys.*, **43**, 474.

WIEST, W. G. AND HEIDELBURGER, C.—(1953) *Cancer Res.*, **13**, 255.

# THE INTRACELLULAR METABOLISM OF 3:4 BENZPYRENE: FURTHER EXAMINATION OF THE SUPERNATANT FRACTION FROM MOUSE LIVER.

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In a previous paper Calcutt and Payne (1954) gave an account of the determination of benzpyrene metabolites within the four major fractions obtainable from mouse liver homogenate. On that occasion we described the discovery that both the recognised primary oxidation products of 3:4 benzpyrene are formed in the supernatant fraction. Since then we have further examined this fraction in the attempt to determine whether either or both these metabolites are produced in any one subfraction.

The problem is difficult as the residue after the removal of cell particulates has never received the same attention as the other fractions. The material in question undoubtedly contains a large number of proteins in solution, as has been shown in electrophoretic studies by Sorof and Cohen (1951*a*, 1951*b*) and other authors. Ultracentrifuge studies by the same authors were also reported and confirmed the findings. Evidence indicating the presence of a large number of enzymes within this fraction has also been summarised by Hogeboom, Schneider and Striebich (1953). Obviously, with such a complex mixture the attempted separation of chemically identifiable components would be a lengthy and tedious process. So, as a first step in the problem, we have attempted a simple subdivision of the supernatant fraction to investigate whether the substrates concerned in the formation of the two benzpyrene metabolites can be separated.

## METHODS.

Two techniques have been used for the subdivision of the supernatant, one, an ultracentrifugation method and the other a simple salting-out process with ammonium sulphate. Purification and estimation of the benzpyrene metabolites were by the same methods as described in a previous paper (Calcutt and Payne, 1954).

The ultracentrifugation technique was the same as that used by Sorof, Golder and Ott (1954) in their studies of protein bound azo dyes in rat liver supernate. After preparative angle ultracentrifugation at an average force of 152,000 $\times$ g. for 7½ hours in the Spinco Ultracentrifuge these authors obtained three fractions based upon the picture obtained by use of the cylindrical lens schlieren optical system. The carcinogenic azo dyes were held for the most part in the fraction representing the bulk of the albumins from rat liver.

Using the supernatant fraction obtained after removal of the cell particulate fractions from a homogenate of mouse liver in Tyrode solution we endeavoured

to repeat the American experiments. Ultracentrifugation under refrigerated conditions was carried out for 7½ hours, using the 26° angle head of the Spinco Model E Ultracentrifuge at a speed of 50,740 r.p.m. (average force 152,000 × g.). We were unable to check the sedimentation by schlieren optical methods but visual inspection showed three distinct zones in the tubes. Under ordinary visible light they appeared as a colourless surface layer, a faintly straw-coloured middle zone and a deeper straw-coloured bottom layer. Examined by the illumination from a U.V. lamp with a Woods glass filter the top layer fluoresced blue, the middle, blue-green and the bottom greenish. Under neither condition was there any indication of sharp demarcation of the three zones. Based on these appearances we separated into three portions the supernatants obtained from livers taken from mice which had been intravenously injected with colloidal benzyprene. Homogenisation and removal of the particulate fractions was as described earlier by Calcutt and Payne (1954).

In a second series of experiments the supernatant fraction was split into two subfractions by salting-out by the addition of an equal amount of saturated ammonium sulphate solution. This gives an approximate separation of the globulins from the albumins. The more efficient methods using ethanol or acetone at lowered temperatures were precluded in the present case because of the risk of altering the distribution of any benzpyrene or its derivatives present.

## RESULTS.

For convenience we have labelled the three layers obtained in the ultracentrifugation experiments as: top layer, middle layer and bottom layer. These three fractions appear to be approximate equivalents of the more accurately designated fractions obtained by Sorof, Golder and Ott (1954). The distribution of benzpyrene and its metabolites over these fractions at intervals up to 22 hours after introduction of the carcinogen is shown in Table I. Two interesting points arise

TABLE I.—*Benzpyrene Metabolites occurring in Subfractions of Supernatant from Mouse Liver after Intravenous Injection of* 3 : 4 *Benzpyrene.*

| Number of mice used. | Time between injection and killing (hours). | Findings. | | |
|---|---|---|---|---|
| | | Top layer. | Middle layer. | Bottom layer |
| 6 | 2 | Bp ; $BpX_2$ ; $BpF_2$ | Bp ; $BpX_2$ ; $BpF_1$ | Bp ; $BpX_1$ ; $BpF_1$ |
| 9 | 4 | Bp ; $BpX_2$ | Bp ; $BpX_1$ ; $BpX_2$ | Bp ; $BpF_1$ |
| 9 | 8 | Bp ; $BpX_2$ | Bp ; $BpX_2$ | Bp ; $BpX_1$ ; $BpF_1$ |
| 8 | 12 | Bp ; $BpX_2$ ; $BpF_2$ | Bp ; $BpX_2$ ; $BpF_1$ | Bp ; $BpF_1$ |
| 9 | 17½ | $BpX_2$ | $BpX_2$ ; $BpF_2$ | $BpX_1$ ; $BpF_1$ |
| 9 | 22 | $BpX_2$ ; $BpF_2$ | $BpX_1$ ; $BpX_2$ $BpF_1$ ; $BpF_2$ | Bp ; $BpX_1$ ; $BpF_1$ |

All fractions were extracted in Tyrode solution.

Abbreviations used: Bp. = 3 : 4 benzpyrene.
$BpX_1$ = 8($OR_1$) − 9(OH) − 8,9-dihydro 3 : 4 benzpyrene.
$BpX_2$ = 8($OR_1$) − 9($OR_2$) − 8,9-dihydro 3 : 4 benzpyrene.
$BpF_1$ = 8($OR_1$) 3 : 4 benzpyrene.
$BpF_2$ = 8.OH 3 : 4 benzpyrene.

here. Firstly, benzpyrene itself has been found in all three subfractions at time intervals up to 12 hours after the injection. This is in distinct contrast to our

previous failure to find the unchanged hydrocarbon in the supernatant fraction. Secondly, the only metabolites in the top layer are $BpX_2$ and its dehydration product $BpF_2$, whilst in the bottom layer there is only $BpX_1$ and its breakdown products $BpF_1$ and $BpF_2$. The middle layer appears as if it were a mixture of the top and bottom layers.

To test whether these distributions were truly representative of intracellular behaviour and not artefacts arising during the separation processes a further series of experiments was carried out. Corresponding subfractions of the supernatant from untreated mouse liver were prepared, mixed with colloidal benzpyrene and incubated at 37° C. in the dark. After varying incubation periods examinations for metabolites were carried out as previously. The findings are detailed in Table II. These are in agreement with those from the *in vivo* experiments and show that metabolism is at the sites found.

TABLE II.—*Benzpyrene Derivatives found after Incubation of Colloidal Benzpyrene with Subfractions of Mouse Liver Supernatant.*

| Number of animals. | Hours of incubation. | Findings. | | |
|---|---|---|---|---|
| | | Top layer. | Middle layer. | Bottom layer. |
| 9 | 4 | $BpX_2$ | $BpX_2$; $BpF_1$ | $BpF_1$; $BpF_2$ |
| 8 | 10½ | $BpX_2$ | $BpX_1$; $BpX_2$; $BpF_2$ | $BpX_1$; $BpF_1$ |
| 12 | 15 | $BpX_2$; $BpF_2$ | $BpX_1$; (trace)<br>$BpX_2$; (trace) | $BpF_1$; $BpF_2$ |

All extractions and incubations were done in Tyrode solution. Abbreviations as in Table I.

In the second series of experiments two subfractions were obtained from the supernate by salting out. These we have designated as: precipitated fraction (roughly representing the globulins) and soluble fraction (roughly representing the albumins). Recovery of benzpyrene metabolites from these two fractions was quantitatively very poor so no further subdivision was attempted. The reasons for the bad recoveries are at present unknown. Results for a series of experiments are given in Table III whilst those obtained in a corresponding series of *in vitro*

TABLE III.—*Benzpyrene Metabolites in Mouse Liver Supernate Subfractions Salted-out with 50 per cent Ammonium Sulphate.*

| Number of mice used. | Time between injection and killing (hours). | Findings. | |
|---|---|---|---|
| | | Precipitated fraction. | Soluble fraction. |
| 10 | 2½ | $BpX_1$; $BpX_2$ | Too weak for identification |
| 12 | 5 | $BpX_1$ | $BpX_2$ |
| 11 | 12 | $BpX_1$; $BpX_2$ | $BpX_2$; $BpF_1$; $BpF_2$ |
| 9 | 17 | $BpX_2$; $BpF_1$ | $BpX_2$; $BpF_1$; $BpF_2$ |

All extractions were done in Tyrode solution. Abbreviations as in Table I.

experiments are given in Table IV. Whilst no clear-cut separation is apparent it seems that the formation of $BpX_1$ is mainly associated with the precipitated fraction whilst $BpX_2$ formation is essentially concentrated in the soluble fraction.

TABLE IV.—*Benzpyrene Derivatives obtained after Incubation of Mouse Liver Supernate Subfractions with the Colloidal Hydrocarbon*

| Number of mice used. | Incubation time in hours. | Findings. Precipitated fraction. | Findings. Soluble fraction. |
|---|---|---|---|
| 12 | 2 | $BpX_1$; $BpX_2$ | $BpX_2$ |
| 12 | 5 | $BpX_1$; $BpF_1$ | $BpX_2$ |
| 12 | 12 | $BpX_1$; $BpF_1$; $BpF_2$ | $BpF_1$ (trace) $BpX_2$; $BpF_2$ |

All extractions were done in Tyrode solution. Abbreviations as in Table I.

Considering the results of the two sets of experiments together it appears reasonable to conclude that different substrates are involved in the formation of $BpX_1$ and $BpX_2$ respectively. That interacting with benzpyrene to produce $BpX_1$ is probably a material having a high molecular weight and capable of being salted out of solution with 50 per cent ammonium sulphate. On the other hand $BpX_2$ appears to arise from action involving a substance or substances of lower molecular weights than in the previous case and also soluble in strong salt solutions.

## DISCUSSION.

Assessment of the results detailed above in relation to the original cell structure is not possible until far more is known about the origin of the various components of the supernatant fraction. In the case of the azo dyes, Sorof, Golder and Ott (1954) found the bulk of bound dyestuff to be associated with the fraction they labelled as the 3·6 proteins. Roughly, this corresponds with our " middle layer." Quantitative estimates of the relative amounts of benzpyrene and metabolites in our fractions have not been possible because of losses during the extraction and purification proceedings, so direct comparison is excluded. On the other hand our experience with respect to the recoveries obtained does not lead us to consider that there is any predominance of metabolism in any one fraction.

During the present experiments unchanged benzpyrene has been found in the three fractions obtained by ultracentrifugation, but only up to about 12 hours after the initial injection. In earlier experiments where the supernatant fraction was examined shortly after the death of the animal the unchanged hydrocarbon could not be detected. In the present series because of the prolonged centrifugation process there has been considerable delay before the fractions have been examined. This suggests that benzpyrene becomes bound to some component of the living cell and is "masked" against detection by absorption spectroscopy. Later this masked benzpyrene is freed, possibly as a consequence of some process of autolysis occurring within the fraction. This corresponds with Boyland's (1949) view that benzpyrene becomes linked in tissues " through the K region or phenanthrene double bond and ... that the linkage is not very stable ".

The results obtained so far in these experiments have demonstrated the complexity of the problems involved in the question of carcinogenesis by benzpyrene. Equally, however, they have shown that future progress must depend upon detailed studies concerning the specific components of the tissues concerned. Some such studies have been commenced.

SUMMARY.

1. The supernatant fraction obtained from livers of mice injected with 3 : 4 benzpyrene has been separated by ultracentrifugation into three subfractions.

2. The fastest sedimenting fraction contained the benzpyrene derivative $BpX_1$ and its breakdown products.

3. The slowest sedimenting fraction contained $BpX_2$ and its breakdown products.

4. The intermediate fraction appeared as a mixture of the other two, containing $BpX_1$ and $BpX_2$.

5. At intervals up to 12 hours after the injection of the hydrocarbon unchanged benzpyrene occurred in all three fractions.

6. Salting-out of the supernate with 50 per cent ammonium sulphate gave results suggesting that $BpX_1$ is mainly associated with the globulin fraction whilst $BpX_2$ is mainly associated with the albumins.

## REFERENCES.

BOYLAND, E.—(1949) *Ann. Rev. Biochem.*, **18**, 217.

CALCUTT, G. AND PAYNE, S.—(1954) *Brit. J. Cancer*, **8**, 554.

HOGEBOOM, G. H., SCHNEIDER, W. C. AND STRIEBICH, M. J.—(1953) *Cancer Res.*, **13**, 617.

SOROF, S. AND COHEN, P. P.—(1951*a*) *J. biol. Chem.*, **190**, 311.—(1951*b*) *Cancer Res.*, **11**, 376.

*Idem*, GOLDER, R. H. AND OTT, M. G.—(1954) *Ibid.*, **14**, 190.

# BEHAVIOUR OF SERA FROM NORMAL AND TUMOUR RATS IN THE IODINE-AZIDE REACTION AND IN POLAROGRAPHIC PROTEOSE TESTS.

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RASCHIG (1915) demonstrated that sulphur compounds catalyse the decomposition of sodium azide dissolved in iodine-potassium iodide solution according to the equation :

$$2\ N_3^- + I_2 \longrightarrow 3\ N_2 + 2I^-.$$

Later, microchemical tests for various sulphur compounds based on this reaction were proposed by Feigl (1939) who also showed that keratin fibres on account of their sulphur-containing amino acids could liberate nitrogen from the reagent. Løvtrup (1951) and Whitman and Whitney (1953) studied manometrically the reaction between iodine-azide reagent and cystine, cysteine and related compounds.

Disturbances are known to occur in the blood proteins of cancerous animals (Winzler, 1953) and some diagnostic tests have been based on these abnormalities. For instance, the original polarographic test devised by Brdička (for review see Kolthoff and Lingane, 1953) demonstrated that cancer sera contained smaller amounts of sulphur proteins than normal sera. It has now been found that sera from tumour-bearing rats are generally less effective than normal rat sera for catalysing the decomposition of iodine-azide reagent as measured manometrically, and it has been shown that there is a high negative linear correlation between tumour size (expressed as percentage of total body weight) and the power of sera from host animals for catalysing iodine-azide decomposition.

Concurrently with these catalytic studies, sera were examined polarographically by a modified Brdička reaction (as carried out by Robinson, 1948). In our hands, this particular polarographic reaction for cancer proved to be statistically unreliable, no significant difference being found between proteose wave heights for tumour and normal sera, nor was any correlation found between proteose wave height and tumour size. Statistical analysis of the results of Munro and Boyd (1951) showed that these authors obtained a positive linear correlation (+ 0·53, $t = 3{\cdot}88$, highly significant at 0·1 per cent level) between tumour weight and proteose wave height in the Waldschmidt-Leitz polarographic test (a procedure which does not involve alkaline denaturation of serum but simply sulphosalicylic acid deproteinisation). Sera from a small group of our animals were examined by a modified Waldschmidt-Leitz test as well as by Robinson's (1948) method and some examples illustrating the differences between the two tests are given here.

As already mentioned, no correlation was found between proteose wave height (Robinson test) and tumour percentage. Nor could proteose be correlated

with catalytic nitrogen output for cancer sera. However, a significant linear negative correlation was observed between proteose wave height and the catalytic activity of normal sera in the iodine-azide reaction.

Some miscellaneous observations have been made of the behaviour of fresh rat serum, oxalated whole blood and plasma in the iodine-azide reaction and an unexpected result was obtained when blood, plasma and serum from the same rat were compared manometrically. While whole blood had a greater catalytic activity than serum, the activity of plasma was generally less than that of serum volume for volume.

## MATERIALS AND METHODS.

### *Animals.*

The transplant rats (albino, Wistar strain) had been inoculated about 14 days previously with dibenzanthracene sarcoma (Rb/3) or with Walker (W) tumour subcutaneously in the right flank. Some specimens of a 20-methylcholanthrene-induced tumour were also available for study. This tumour, which grew more slowly than the Rb/3 or W transplants and was less liable than these to necrosis, was studied in transplants of age 27 and 41 days. Tumour animals and normal controls were maintained on a bread-and-milk diet supplemented with horse meat and cod-liver oil separately on alternate days. A number of Walker transplant animals and normals which had been maintained on Edinburgh rat cube No. 86 diet were also studied. No comparison between these diets in relation to the effects described is intended and the results have been analysed without regard to kind or age of tumour but merely to tumour size.

### *Serum collection.*

Under ether anaesthesia, the rat's chest is opened, the aorta severed and blood collected in a centrifuge tube. After centrifugation for 10 min. at 2000 r.p.m., serum was pipetted off and stored in an open pyrex tube overnight at 0° unless otherwise stated. It is interesting that 22 of 32 tumour sera exhibited various degrees of turbidity while only 3 of 31 normal sera showed very faint turbidity. Perhaps this finding is related to effects already described by Green and Jenkinson (1934) in their study of the reduced esterase activity of cancer sera. Often tumour rat sera are as intensely opalescent as those described by Rosenthal (1949) in another connection. In the present work no direct correlation between tumour size and degree of opalescence was evident though some observations suggest that the effect might be related to extent of necrosis.

### *Manometry.*

A stock solution of 0·1 N iodine (A.R.) in 4 per cent potassium iodide (A.R.) was prepared and standardised by thiosulphate titration. Sodium azide (3 g.; B.D.H. Reagent) was dissolved in 0·1 N iodine solution and made up to 100 ml. These solutions were stored at 0°.

Chilled sera were allowed to attain room temperature and 0·1 ml. samples were pipetted into pyrex tubes. Distilled water (5 ml.) was added to each tube and the contents mixed by shaking gently. Iodine-azide reagent (2 ml.) was placed in the main vessel of a respirometer flask and an 0·5 ml. sample (≡ 0·01 ml. serum; nitrogen output at 100 minutes is directly proportional to the amount of serum added per

flask at least up to 0·02 ml. serum per flask) of serum dilution was added to the side arm of the vessel. The flasks were then connected to their manometers and shaken (120 oscillations per minute) in a thermostat bath at 25°. After equilibration for 20 minutes in the presence of air, the respirometer flask contents were mixed and nitrogen output was measured in the usual way over a two-hour period. Each experiment was controlled by a blank consisting of iodine-azide reagent (2 ml.) and distilled water (0·5 ml.). On a few occasions, the control showed an output of about 5 $\mu$l. of gas, but this was not taken into account in the experimental results. Controls consisting of (*i*) 3 per cent sodium azide in 4 per cent KI only (2 ml.) + serum dilution (0·5 ml.) and (*ii*) 0·1 N iodine in 4 per cent KI (2 ml.) + serum dilution (0·5 ml.) showed no gas output or uptake under the experimental conditions.

*Polarography.*

Method A (based on Robinson, 1948). Serum (kept 24 hours at 0°) is allowed to attain room temperature and an 0·4 ml. sample is mixed with 0·1 N potassium hydroxide (1 ml.). After 45 minutes 20 per cent sulphosalicylic acid (1 ml.) is added, the mixture vigorously shaken and, after 10 minutes exactly, filtered through fluted Whatman No. 50 filter paper (9 cm.). The clear filtrate (0·5 ml.) is mixed with 5 ml. of the hexammine cobaltic chloride-ammonia buffer mixture recommended by Robinson (1948) just prior to polarography.

Polarograms were recorded photographically by means of a Cambridge polarograph. As reference anode, a saturated calomel electrode (S.C.E.) was connected to the test serum mixture by means of agar bridges (3 per cent agar agar in saturated KCl). The dropping mercury electrode had the following characteristics: at 20°, in 0·1 N KCl (open circuit) with head of mercury = 50 cm., m = 0·003118 g./sec., and $t$ = 1·83 sec. Capillary radius = 22·9 $\mu$ (for explanation of terms see Kolthoff and Lingane, 1953). Polarograms were recorded with galvanometer sensitivity = 1/150 and zero damping between — 0·8 and — 1·9 volts. Each polarogram showed an inflection at — 1·2 volts which was used as base line marker for determining the height of the catalytic proteose wave, the peak of which occurs at about — 1·58 volts versus S.C.E. The proteose wave heights (mm.) for tumour and normal sera are collected in the histograms of Fig. 3.

Method B (based on Waldschmidt-Leitz; compare Munro and Boyd (1951)). 0·5 ml. samples of sera were treated with 1 ml. 25 per cent sulphosalicylic acid and distilled water (1 ml.) and the well-shaken mixture centrifuged for 10 min. at 2000 r.p.m. The supernatant was filtered through Whatman No. 50 filter paper. It may be of interest to note that, after storage at 0°, the clear filtrates from normal blood often became cloudy, sometimes with a white precipitate separating, while those from tumour blood remained quite clear. Fresh filtrate (0·5 ml.) was added to Robinson's cobalt buffer mixture (5 ml.) and polarographed as described under method A except that the galvanometer sensitivity now employed was 1/300.

## RESULTS.

*Catalysis of iodine-azide decomposition by tumour and normal rat sera.*

Typical nitrogen evolution curves are shown in Fig. 1 from which it is seen that after 100 minutes nitrogen evolution has practically ceased. The volume of nitrogen evolved at this time is calculated in the usual way (Umbreit, Burris and

Stauffer, 1951) and the value ($100'N$) taken as a measure of the catalytic activity of the serum. Azide decomposition appears to follow a first order reaction since plots of $\log_{10}\left(\frac{N_\infty - N_T}{N_\infty}\right)$ against time ($T$) are linear (here, the $100'N$ value is taken as $N_\infty$).

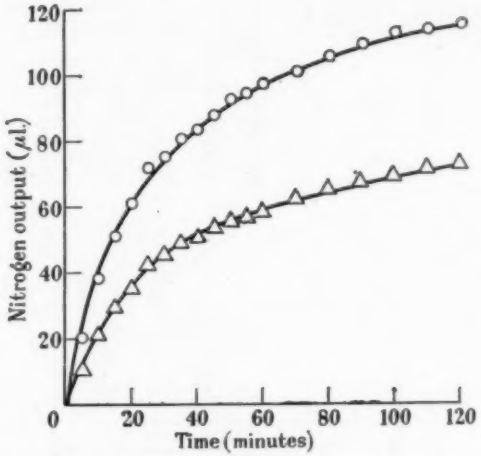



FIG. 1.—Decomposition of iodine-azide reagent catalysed by tumour rat serum (—△— : tumour per cent = 20·9) and by normal rat serum (—○—).

Fig. 2 shows the distribution of serum $100'N$ values for tumour and normal rats. There is a considerable overlap between tumour and normal group values so that a diminished capacity of serum for promoting iodine-azide decomposition is not specific for cancer.

*Polarography of tumour and normal rat sera.*

Histograms for proteose wave heights in tumour and normal groups are given in Fig. 3. Evidently, the polarographic filtrate test (Method A) is rather unsatisfactory as a guide to the pathological condition of the animals. In Fig. 4 are shown some comparative results for sera from tumour and normal animals examined by polarographic Methods A and B. In Test B, 8 tumour animals (6W (5 ♂) and 2 Rb/3 (1 ♂)) gave a mean proteose wave height of 47·4 mm. (S.D. = 4·23) and 6 normal rats (2 ♂) gave a mean wave height of 34·1 mm. (S.D. = 3·53). When tumour per cent was compared with wave height a negative linear correlation of — 0·39 ($r$) was obtained ($t = \frac{r\sqrt{n-2}}{\sqrt{1-r^2}}$, where $n$ = number of animals. Here $t = 0{\cdot}95$, not significant). It is interesting that Munro and Boyd (1951) found a positive correlation between tumour weight and proteose wave height. Their sample was much larger than the present one.

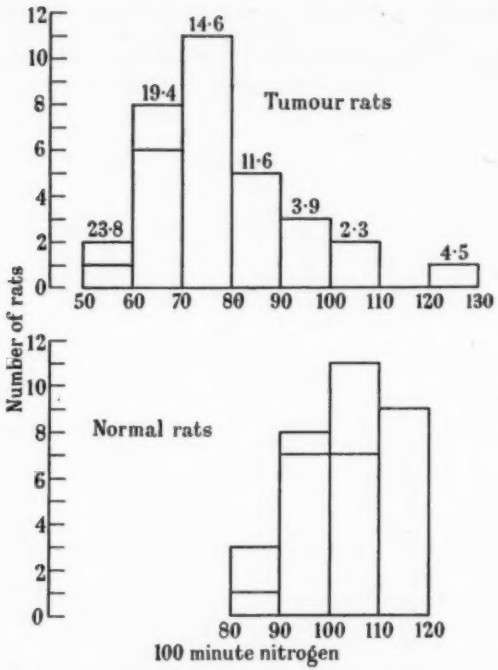



FIG. 2.—Histograms showing the distribution of serum 100′*N* values for tumour and normal rats. The number at the head of each class column is the mean percentage tumour weight for the animals in the class. Horizontal subdividing lines in the classes indicate that animals above the line are those which received Diet No. 86.

| Kind and number of rats. | *n* | Mean 100′*N*. | S.D. | Difference of means. | S.E.* |
|---|---|---|---|---|---|
| Tumour (T) | 32 | 77·85 | 14·62 | 27·84 | 3·05 |
| Normal (N) | 31 | 103·69 | 8·99 | | |

* Standard error given by $\sqrt{\frac{(S.D._T)^2}{n_T} + \frac{(S.D._N)^2}{n_N}}$.

Difference significant.

*Statistical correlations.*

Analyses were carried out as described by Bradford Hill (1952) and Moroney (1953). Sera from normal rats ($n = 31$) showed a highly significant negative linear correlation coefficient between their 100′*N* and proteose (Method A) values (— 0·65, $t = 4·62$, highly significant at 0·1 per cent level). On correlating log 100′*N* with log proteose a coefficient of — 0·63 was obtained. However, when 100′*N* and proteose values for tumour animals were compared a small negative correlation coefficient (— 0·04) was obtained which was not significant ($t = 0·20$).

A highly significant negative linear correlation coefficient was observed when tumour serum 100′*N* values were compared with tumour weights (— 0·55, $t = 3·55$,

significant at 1 per cent level) and an even greater degree of correlation appeared when percentage tumour weights were used instead of absolute weights for comparison (— 0·71, $t = 5·55$, highly significant at 0·1 per cent level). Higher correlation coefficients were obtained when log $100'N$ values were plotted against log tumour weight (— 0·69, $t = 5·24$) or log tumour per cent (— 0·77, $t = 6·60$, highly

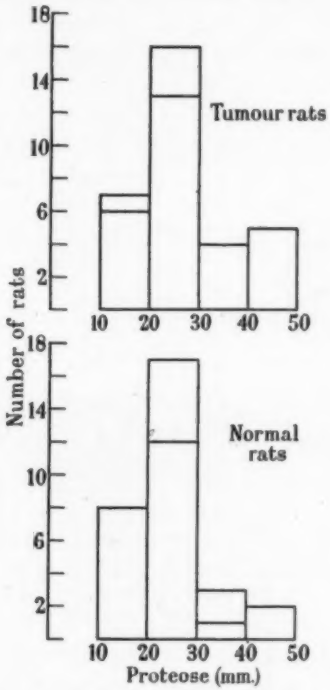



FIG. 3.—Histograms showing distribution of serum polarographic proteose values (Method A) for tumour and normal rats.

| Kind and number of rats. | $n$ | Mean proteose (mm.). | S.D. | Difference of means. | S.E.* |
|---|---|---|---|---|---|
| Tumour (T) | 32 | 27·39 | 8·41 | 2·75 | 1·88 |
| Normal (N) | 31 | 24·64 | 6·44 | | |

* Standard error given by $\sqrt{\frac{(S.D._T)^2}{n_T} + \frac{(S.D._N)^2}{n_N}}$.

Difference not significant.

significant at 0·1 per cent level). The scatter diagram for the last-mentioned relation is shown in Fig. 5.

When proteose values for tumour animals were compared with tumour per cent and with tumour weight small negative linear correlation coefficients were obtained, namely, — 0·11 and — 0·15 which were not significant ($t$ tests = 0·64 and 0·83 respectively).

*Miscellaneous observations.*

(i) *Fresh serum and oxalated whole blood as catalysts for iodine-azide decomposition.*—Whole blood (2 mg. potassium oxalate per ml.) and serum were obtained from 14 transplant animals (11 W (3 ♂) and 3 Rb/3 (1 ♂)). 0·1 ml. samples of each were mixed with 5 ml. of distilled water and 0·5 ml. aliquots of the dilutions were used for manometric work. It was first ascertained that potassium oxalate at the level introduced with the blood had by itself no influence on iodine-azide decomposition. The results are summarised in Table I.

TABLE I.—*Fresh Rat Serum and Oxalated Whole Blood from Tumour-bearing and Normal Animals as Catalysts for Iodine-azide Decomposition.*

| Number and kind of rats. | Mean $100'N$ (μl.) for | | S.D. for | | Correlation between tumour per cent and | |
|---|---|---|---|---|---|---|
| | Serum. | Whole blood. | Serum. | Whole blood. | Serum. $100'N$. | Whole blood. $100'N$. |
| 14 Tumour* | 91·93 | 133·12 | 13·43 | 26·02 | −0·81† | −0·79‡ |
| 8 Normal | 132·64 | 172·49 | 15·41 | 8·99 | — | — |

* Tumour per cent range is 0·3 to 18·2; mean = 8·32; S.D. = 6·04.
† $t$ test = 4·77, highly significant at 0·1 per cent level.
‡ $t$ test = 4·40, significant at 1 per cent level.

As was expected, whole blood was more effective than serum for promoting azide decomposition. Fresh serum (both tumour and normal) had a higher catalytic activity than 24-hour-old serum (cf. Tables I and II). Some experiments indicated that tumour sera lost slightly their capacity for catalysis (referred to 24-hour-old serum activity) whereas normal sera gained in this respect when they were preserved for several days at 0°. In some experiments with fresh whole blood and serum the difference between the catalytic activities tended to be greater for tumour blood than for normal blood.

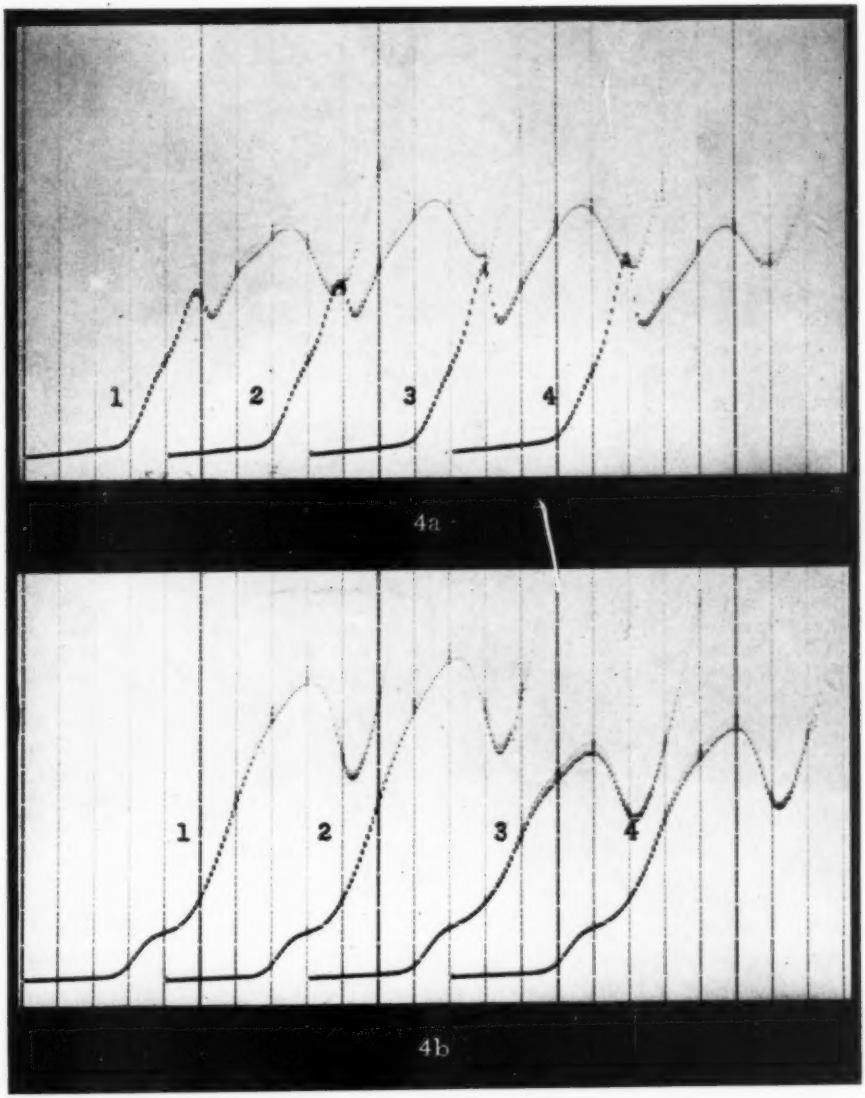
TABLE II.—*Comparison of the Catalytic Activities of Oxalated Whole Blood, Plasma and Serum from Tumour-bearing and Normal Rats in the Iodine-azide Reaction.*

| Experiment number. | Animal. | Tumour (%). | $100'N$ output (μl.) | | |
|---|---|---|---|---|---|
| | | | Whole blood. | Plasma. | Serum. |
| 1 | Rb/3 | 3·4 | 168·1 | 77·4 | 104·3 |
| | Rb/3 | 18·4 | 126·8 | 80·9 | 96·3 |
| | Normal | — | 163·0 | 104·1 | 125·7 |
| 2 | Rb/3 | 15·9 | 140·9 | 96·8 | 105·1 |
| | Rb/3 | 4·5 | 178·4 | 131·3 | 119·4 |
| | Normal | — | 183·0 | 112·5 | 122·0 |

## DESCRIPTION OF PLATE.

FIG. 4.—Polarograms illustrating the difference between the Robinson Test (Method A) and the Waldschmidt-Leitz Test (Method B).

Upper curves, Method A; lower curves, Method B. Each polarogram begins at − 0·8 volts applied. Abscissa interval = 0·1 volt. Serum No. 1 from Rb/3 (tumour per cent = 19·3). Serum No. 2 from Rb/3 (tumour per cent = 16·3). Serum No. 3 from normal rat. Serum No. 4 from normal rat.


1
2
3
4
4a
1
2
3
4
4b

(ii) *Effect of heating serum dilutions.*—Heat treatment (five minutes in a boiling water bath) increased the capacity of serum dilutions for catalysing nitrogen outnut and there was a suggestion that the increased capacity is greater for normal than for tumour sera.

(iii) *Comparison of the behaviour of fresh rat whole blood plasma and serum in the iodine-azide reaction.*—In the experiments given in Table II, the catalytic activities were determined three hours after collection of the fluids. The behaviour

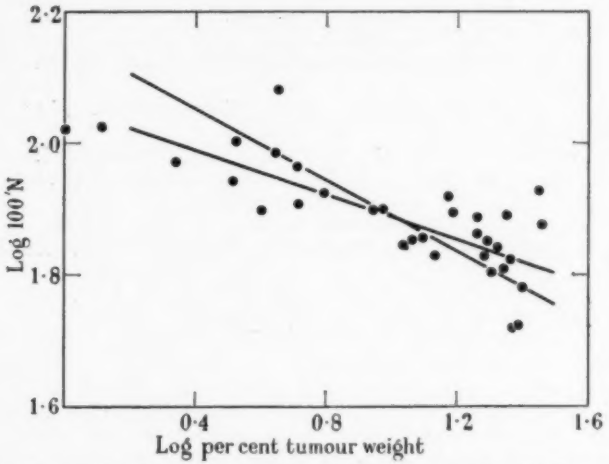



FIG. 5.—Scatter diagram showing negative linear correlation between log 100′$N$ and log percentage tumour weight.

The regression equations were calculated in the usual way (e.g., Moroney, 1953) and are as follows:

Log tumour per cent = − 3·5038 log 100′$N$ + 7·6332.
Log 100′$N$ = − 0·1690 log tumour per cent + 2·0582.

The regression lines are shown in the figure.

of plasma was unexpected in that it had a lower catalytic activity than the serum except in one case. It would appear that fibrinogen reduces the activity of serum proteins in the iodine-azide reaction. In some experiments with normal rabbit whole blood and serum it was found that the latter had a greater catalytic activity than the former. It has not yet been possible to determine whether we are dealing with species differences or with effects of ageing of the blood fluids.

## SUMMARY.

Sera from rats bearing various transplant tumours have generally much reduced capacities for catalysing the decomposition of iodine-azide reagent as compared with normal sera. There is a highly significant negative linear correlation relating log tumour per cent and log 100′$N$. Similar correlations hold for oxalated whole blood and fresh serum from tumour-bearing rats.

A polarographic proteose test employed as a " control " for the iodine-azide reaction proved to be statistically unreliable. Only very small correlations (with-

out significance) were found between tumour sera proteose wave heights and tumour percentage or serum $100'N$ values. However the test did show a significant linear negative correlation between serum proteose and $100'N$ values for normal animals.

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## REFERENCES.

Bradford Hill, A.—(1952) ' Principles of Medical Statistics,' 5th ed. London (The Lancet, Ltd.).

Feigl, F.—(1939) ' Qualitative Analysis by Spot Tests,' 2nd ed. New York (Nordeman Publishing Co. Inc.).

Green, H. N., and Jenkinson, C. N.—(1934) *Brit. J. exp. Path.*, **15**, 1.

Kolthoff, I. M., and Lingane, J. J.—(1953) 'Polarography', **2**, pp. 849–876, 2nd ed. New York and London. (Interscience Publishers).

Løvtrup, S.—(1951) *C.R. des trav. du Lab. Carlsberg, Sér chim.*, **27**, 63.

Moroney, M. J.—(1953) 'Facts from Figures,' pp. 271–319, 2nd ed. London (Penguin Books, Ltd.).

Munro, L. A., and Boyd, D. J.—(1951) *Canad. J. Chem.*, **29**, 1115.

Raschig, F.—(1915) *Ber. dtsch. chem. Ges.*, **48**, 2088.

Robinson, A. M.—(1948) *Brit. J. Cancer*, **2**, 360.

Rosenthal, R. L.—(1949) *Science*, **110**, 43.

Umbreit, W. W., Burris, R. H., and Stauffer, J. F.—(1951) ' Manometric Techniques and Tissue Metabolism,' 2nd ed. Minneapolis (Burgess Publishing Co.).

Whitman, D. W., and Whitney, R. M.—(1953) *Analyt. Chem.*, **25**, 1523.

Winzler, R. J.—(1953) *Plasma Proteins in Cancer in Advances in Cancer Research*, **1**, 503.

# SURVEY OF PAPERS.

SPENCER presents data from fifteen countries in four continents emphasising the importance of local factors in accounting for known variations of cancer incidence. Iodine availability, as indicated by goitre incidence, appears to be one such factor. The relationship of thyroid deficiency to cancer incidence is discussed in connection with other known hormonal influences, particularly those of pituitary origin (p. 393).

STERN gives an account of the post-mortem findings in 87 cases of carcinoma of the bronchus, describing in greater detail those cases with solitary cerebral metastases unaccompanied by visceral deposits (p. 412).

PHILPS reports on two patients with adenocarcinoma in whose sputum the tumour cells were identified (p. 420).

PHILPS describes and illustrates some appearances seen in sputum films which may erroneously be thought to be due to carcinoma (p. 422).

LEVEAUX discusses the diagnosis of carcinoma of the pancreas in the light of the findings in 46 proven cases and proposes a scheme of investigation (p. 427).

LUMB reports the results of transplanting sixty different human tumours into mouse brain, and describes the histological appearances of the transplants during the first 28 to 35 days after inoculation (p. 434).

PULLINGER describes the mammary carcinomas which developed in 9 out of 27 unmated RIIIb female mice without milk factor following treatment of the second and third nipple areas by local surface and penetrating X-irradiation. Spontaneous carcinoma does not occur in unmated females of this strain and breast tumours are found in less than 2 per cent of breeders. The tumours produced are associated with atrophy of the breast tissue (p. 445).

HORNING and WHITTICK describe the multiple, bilateral renal tumours which develop in intact male golden hamsters following the subcutaneous implantation of a 20 mg. pellet of diethylstilboestrol. They find that the renal tumours arise from the epithelium of the cortical tubules and grow both by expansion and infiltration. An account is also given of the associated oestrogenic changes in other organs of the body (p. 451).

SCHOENTAL, HEAD and PEACOCK give an account of the changes in the liver, ranging from hyperplasia to neoplasia, found in the majority of a series of rats treated for 10 months or more with the Senecio alkaloids retrorsine and isatidine by mouth (p. 458).

MILLER and PYBUS have investigated the incidence of spontaneous lung tumours in two inbred strains of mice and their reciprocal hybrids and compared the results with the higher incidence of lung tumours induced in the same material by subcutaneous injection of methylcholanthrene (p. 466).

MILLAR and NOBLE give an account of the morphology and growth characteristics of a line of mammary fibro-adenomata in rats in the course of twelve generations of transplantation. The variations which occurred, including changes in the proportion of the epithelium to connective tissue, development of fibromata and fibrosarcomata, and bony or cartilagenous metaplasia, are described, as also are the effects of endogenous hormones (p. 485).

MILLAR and NOBLE give an account of the effects of a variety of exogenous hormones on the growth characteristics and morphology of a transplanted mammary fibro-adenoma of the rat (p. 495).

MILLAR and NOBLE give an account of the pathology and growth characteristics of tumour lines originating from fibrosarcomatous tissue occurring in transplanted rat mammary fibro-adenomata. They find that diethylstilboestrol in large doses causes only slight retardation of tumour growth, contrasting with the arrest or marked retardation of growth of the fibro-adenomata under the influence of oestrogen (p. 508).

JOLLES and LAWS find that the administration of Synkavit to rabbits prior to and after irradiation slightly increases their tolerance of irradiation, whereas in rats an opposite effect is found (p. 513).

COHEN and COHEN find that when the $C_3H$ mammary carcinoma is irradiated before implantation into reciprocal $F_1$ hybrid hosts, the $LD_{50}$ does not differ significantly between the presumably factor-free hybrids and the factor-harbouring reciprocal hosts, and that the values do not differ significantly from that for the same tumour in the $C_3H$ parent-strain host (p. 522).

POWELL has studied the effects of a series of dithiocarbamates on embryonic mouse fibrocytes and sarcoma cells *in vitro*. He finds that sodium diethyldithiocarbamate is the least toxic of these compounds and is more toxic to sarcoma cells than fibrocytes in rapidly growing cultures (p. 529).

BATHER has studied the growth characteristics of Rous No. 1 sarcoma in the peritoneal cavity of chickens. As few as 100 suspended cells are sufficient to establish an ascites tumour, but cell-free virus fails to do so. Attachment of tumour cells to, and invasion of abdominal organs rapidly follows intraperitoneal inoculation and nearly all the chickens die within a fortnight (p. 535).

BATHER has estimated the cytoplasmic particulate yield, which he equates with the total virus content, from a series of filterable fowl tumours by the biuret colorimetric procedure and finds no significant relationship between infectivity and the number of particles obtained. Particulate yields from a non-filterable sarcoma did not vary with host age or tumour growth rate (p. 545).

CALCUTT and PAYNE have investigated the distribution of benzpyrene metabolites within mouse liver cells treated both *in vivo* and *in vitro* with the carcinogen. Examination showed $BpX_2$ exclusively in nuclei, mitochondria and microsomes at intervals up to 24 hours. $BpX_1$ and $BpX_3$ both occurred in the supernatant fraction (p. 554).

CALCUTT and PAYNE have separated the supernatant fraction obtained from livers of mice injected with 3 : 4 benzpyrene into three subfractions by ultracentrifugation. They find the benzpyrene derivative $BpX_1$ and its breakdown products in the fastest sedimenting fraction, and $BpX_3$ in the slowest, with a mixture in the intermediate fraction. Further study of the supernate suggests that $BpX_1$ is mainly associated with the globulins, $BpX_3$ with the albumins (p. 561).

NEISH finds that sera from rats bearing various transplant tumours have generally much reduced capacities for catalysing the decomposition of iodine-azide reagent as compared with normal sera (p. 566).